Characterization of the molecular and immunological properties of *Acanthocheilonema viteae* tropomyosin.

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I. Summary

The study presented here describes the immunological properties of *Acanthocheilonema viteae* tropomyosin, a muscle associated protein reported to be a promising vaccine candidate. *A. viteae* is a filarial parasite of jirds that is used as a laboratory model for the important human parasite *Onchocerca volvulus*. Our experiments focus on unraveling the functional properties of *A. viteae* tropomyosin in the context of a natural infection, experimental immunization, or vaccination against parasite challenge. Additionally, the allergenic potential of tropomyosin was investigated along with the ability to induce high levels of specific IgE. A part of this study was also aimed at the development of anti-tropomyosin monoclonal antibodies (mAb) and their characterization.

This study revealed that tropomyosin can be a promising antigen for a vaccine against filarial nematodes, however, effective only in a Th1 biased environment. Vaccination with protein or DNA resulted in 30% and 45% protection, respectively, and protection against experimental challenge was not associated with specific IgG or IgE antibodies.

A. viteae tropomyosin is a functional allergen in the course of a natural infection and leads to the production of high levels of specific IgE. By screening of synthetic peptide libraries 13 IgG and 11 IgE co-located epitopes were characterized. Functional assays showed that this protein cross-reacts with tropomyosins of other nematodes and shrimp, and that these proteins share IgE epitopes.

Three mAb were raised against recombinant and native *A. viteae* tropomyosin and for two of them (NR1 and R21) the regions of binding were identified. Specific mAb showed that tropomyosin is abundant on the cuticle of L3 and mf of the parasite. Deglycosylation of the native protein showed that the epitope of the third antibody (N11) appeared to be formed by the posttranslational modifications.

Additionally, immunization of mice showed that *A. viteae* tropomyosin induced a similar pattern of cell activation and antibody production as aluminium hydroxide adjuvant, yet additionally it leads to the induction of IL-10 and the increase of a $GR1^+/CD11b^+$ cell population. These cells are regarded as natural suppressors.

Taken together, these results show that *A. viteae* tropomyosin has immunomodulating properties and can in addition be considered as a component of an efficient vaccine.

II. Zusammenfassung

Die vorliegende Arbeit beschreibt die immunologischen Eigenschaften von *Acanthochilonema viteae* Tropomyosin, einem Muskel-assoziierten Protein, das als vielversprechend für die Vakzinierung gegen Filariosen angesehen wird. *A. viteae* ist ein zu den Filarien gehörender Parasit von Gerbilen und wird als Labormodell für die humanpathogene Filarie *Onchocercha volvulus* genutzt. Diese Arbeit hatte die funktionelle Charakterisierung von *A. viteae* Tropomyosin im Kontext der natürlichen Infektion sowie der experimentellen Immunisierung und Vakzinierung zum Ziel. Zusätzlich wurde das allergene Potential des Tropomyosins und die Produktion von spezifischen IgE-Antikörpern gegen das Protein überprüft. Ein weitere Teil dieser Arbeit beschäftigt sich mit der Entwicklung von Tropomyosin-spezifischen monoklonalen Antikörpern und deren Charakterisierung.

Es konnte gezeigt werden, dass Tropomyosin als vielversprechender Kandidat zur Vakzinentwicklung gegen Filariosen angesehen werden kann, wobei berücksichtigt werden muss, dass deutliche Effekte nur unter Th1-Bedingungen einer Immunantwort erzielt werden konnten. Die Vakzinierung mit Protein oder DNA führte zu um 30 bzw. 45% reduzierten Adultwurmzahlen und dieser Schutz ging nicht mit der Bildung von spezifischen IgG oder IgE Antikörpern gegen Tropomyosin einher.

A. viteae Tropomyosin fungiert im Zuge einer natürlichen Infektion als funktionelles Allergen und führt zur Produktion von hohen Mengen spezifischen IgEs. Durch ein Screening synthetischer Peptid-Bibliotheken konnten 13 IgG- und 11 IgE-Epitope charakterisiert werden. IgE Antikörper gegen *A. viteae* Tropomyosin zeigten in funktionellen Analysen eine Kreuzreaktivität mit aus anderen Nematoden sowie Garnelen gewonnenem Tropomyosin.

Es wurden drei monoklonale Antikörper gegen rekombinant exprimiertes und natives Tropomyosin generiert. Für zwei Klone (NR1 und R21) wurden die Bindungsregionen identifiziert. Mit Hilfe dieser Antikörper konnte gezeigt werden, dass Tropomyosin auf der Oberfläche der Kutikula der Larvenstadien L1 und L3 des Parasiten vorkommt. Durch die Deglykosylierung des nativen Proteins wurde deutlich, dass ein durch den dritten generierten Antikörper (N11) erkanntes Epitop von posttranslationellen Modifikationen gebildet wird.

Schließlich führte die Immunisierung von Mäusen mit *A. viteae* Tropomyosin zu einem ähnlichen Profil der Zellaktivierung und Antikörperproduktion wie das Adjuvant

Aluminiumhydroxid. Die Behandlung führte zu einem Zuwachs einer durch die Oberflächenmoleküle Gr1 und CD11b charakterisierten Zellpopulation und zur IL-10 Produktion in der Milz immunisierter Tiere. Es konnte gezeigt werden, dass $Gr1^{+}CD11b^{+}$ Zellen an der Produktion dieses Zytokins Teil haben.

Zusammenfassend kann gesagt werden, dass *A. viteae* Tropomyosin immunmodulierende Eigenschaften aufweist und zusätzlich als Komponente eines zu entwickelnden Vakzins gegen Filariosen in Frage kommt.

1. Introduction

1.1. Filarial infections

One of the major problems of public health in tropical countries are infections with filarial nematodes witch affect about 120-150 million people (http://www.who.int/inf-fs/en/fact102.html). The forms of filariasis that have the most significant influence on global population health are: lymphatic filariasis caused by *Wuchereria bancrofti* and two species of genus *Brugia*: *B. malayi* and *B. timori*, onchocerciasis (called also “river blindness”) caused by *Onchocerca volvulus*, loiasis caused by *Loa loa* (Hoerauf et al., 2003). Filarial nematodes are classified as a part of a phylum *Nematoda* within a *Filarioidea* superfamily.

Onchocerciasis occurs in 34 African and Latin American countries. It is also reported from the Arabian Peninsula. The vast majority of an estimated 37 million population of people that are infected with *O. volvulus* live in sub-Saharan Africa. Parasite infections have caused blindness in about 270,000 and left another half million with severe visual impairment (Burnham et al., 1998). Onchocerciasis is not only a blinding disease, but in some cases also a chronic systemic illness that causes changes in the immune system. Severe infections can cause symptoms like extensive and disfiguring skin changes, musculoskeletal complaints, weight loss and growth arrest. The disease is endemic in some of the world's poorest areas and its influence on public health has also a major impact on the economy of the regions (Evans et al., 1995).

The life cycle of *O. volvulus* starts when infective L3 larvae (L3) enter the human during the blood meal of an infected female black fly *Simulium*. Within 1-3 months the L3 larvae moult to the L4 and later to adults. Male and female worms dwell in connective tissue and eventually are encapsulated in callagenous nodules, mostly under the skin of a host. Each female worm releases 1,300-1,900 microfilariae per day for 9-11 years (Schulz-Key and Karam, 1986). Microfilariae that migrate out of the nodules into the dermis of the skin are ingested by *Simulium* flies. In an intermediate host microfilariae penetrate the gut and settle in the thoracic flight muscles where they moult and develop into the L2 and subsequently into the infective L3 stage. The manifestation of onchocerciasis in humans is mainly due to the host inflammatory responses directed against dead or dying

microfilariae (they release their endosymbiotic *Wolbachia sp*., bacteria against which, the elements of immune system act, Pearlman and Gillette-Ferguson, 2007). These reactions are responsible for the development of the “river blindness” syndrome. When microfilariae from the skin enter the cornea and conjunctiva a punctuate keratitis develops around dead ones which can clear when inflammation settles. Sclerosing keratitis and iridocyclities are likely to develop after years of prolonged infection causing permanent visual impairment or blindness (Chan et al., 1987). One of the consequences of the disease is also chronic and acute papular onchodermatitis.

So far, strategies to eliminate onchocerciasis have been based on vector control and mass treatment with the anti-microfilaricidal drug Ivermectin (Molyneux et al., 2003) carried out by the Oncocerciasis Control Programme (OCP). The OCP sponsored by several organisations including World Bank and WHO aimed at eradication of onchocerciasis as an important public health disease and as an obstacle for the social and economic development of regions infested with *O. volvulus*. OCP used biodegradable insecticides to eradicate the simulium vector but despite high initial success it faced the rising problem of developing resistance to used chemicals and reinvasion of previously cleared areas by migrating black flies. Invermectin acts as an agonist of the parasite neurotransmitter γ-aminobutyric acid (Soboslay et al., 1987) and induces an influx of Cl^- through channels not regulated by γ-aminobutyric acid. Ivermecitin is an efficient microfilaricidal but unfortunately it has no real effect on adult worms. It affects only temporarily the embryonic development and release of microfilariae from the female (Schulz-Key et al., 1992). Moreover, there are several drawbacks to the chemotherapeutic treatment (Burnham et al., 1998). It is extremely difficult to achieve eradication of parasite infection unless a very costly treatment is sustained for a period of 12 to 15 years (estimated life span of the adult worm). Thus, a more efficient treatment and parasite control approach has been suggested. Concept is based on simultaneous use of a drug and a prophylactic vaccination. There are good reasons to believe that such a combined treatment that acts on both infectious stages of the parasite (mf with a drug and L3 with a vaccine) lead to eradication of onchocerciasis.

Therefore, it is important to characterise antigens playing critical roles in parasite development and transmission that could be targets for an efficient vaccine.

1.2. Filarial proxi models for research

Research in filariasis is dependent on the use of laboratory animal models owing to the inability to carry out experiments on human population. The filarial parasites are host-specific and this is one of the significant limitations that have hindered the functional characterisation of relevant target molecules of *Onchocerca* species. Thus, model systems, that involve the use of parasites in surrogate models, were developed. Study on *Onchocerca spp.* involves the implantation of *Onchocerca spp.* in subcutaneous chambers in CBA/J or DBA/2J mice (Townson and Bianco, 1982; Abraham et al., 1992). A similar approach with *Brugia spp.* / BALB/c mouse system has been used as a chemotherapeutic (Devaney et al., 1985) and immunological (Carlow and Philipp 1987) model for the brugian filariasis. These systems have a major disadvantage because they rely on studies of parasites in non-natural hosts. It can be overcome by the use of natural life-cycle models of the filaria *Brugia pahangi* in cats (serves as a model for *Brugia* and *Wuchereria* in humans; Grenfell et al., 1991), bovine *Onchocerca spp*. or rodent filariae like *Acanthocheilonema viteae* and *Litomosoides sigmodontis* in their natural hosts (plays similar role as *B. pahangi* / cat model but for Onchocerciasis; Abraham et al., 2002). Unfortunately, owing to the lack of adequate immunoreagents the underlying immunological mechanisms can not be investigated fully in these model systems. Recently, a promising model for Onchocerciasis based on full cycle of *L. sigmodontis* in BALB/c mice was developed. Due to the full knowledge of the mouse genetic background, complete and correct development of filarial parasites in that host, it does not have the limitations of previously utilised models.

1.3. The A. viteae proxi model for O. volvulus

A. viteae in its natural host *Meriones unguiculatus* serves as a model for onchocerciasis, the disease caused by *O. volvulus*. *A. viteae* has a very similar life cycle (Fig. 1) and has some parallels to *O. volvulus*. Both reside in the subcutaneous tissue of their hosts and are therefore in the same immunological compartment. However, *A. viteae* parasites do

not form nodules and microfilariae that are present in blood cause no eye lesions like *O. volvulus*. Furthermore, both parasites share an array of antigenic similarities as demonstrated by anti-*O. volvulus* monoclonal antibodies (Nogami et al., 1986), and a high homology in corresponding molecules that have so far been characterised from both parasites. In addition, cross-protection between species has been shown in filariasis (Storey and Al-Mukhtar, 1982; Geiger et al., 1996) so that vaccine candidates established in one system could be tested in others. The *A. viteae* / *M. unguiculatus* system allows the study of resistance to challenge infection following immunisation (Abraham et al., 2002). Interestingly, it has been shown that immunisation with irradiated *A. viteae* L3 led to 90% protection against challenge infection, while immunisation with excretory-secretory products (ESP) led to 70% protection (Lucius et al., 1991). Parallel results were also obtained using irradiated L3 in other filariasis models (Lange et al., 1993; Johnson et al., 1998; Trees et al., 2000) and it was shown in this model that immunisation with irradiated L3 could also lead to resistance against homologous challenge infection (Abraham et al., 2002). Therefore, the *A. viteae* / *Meriones* system was used in this study.

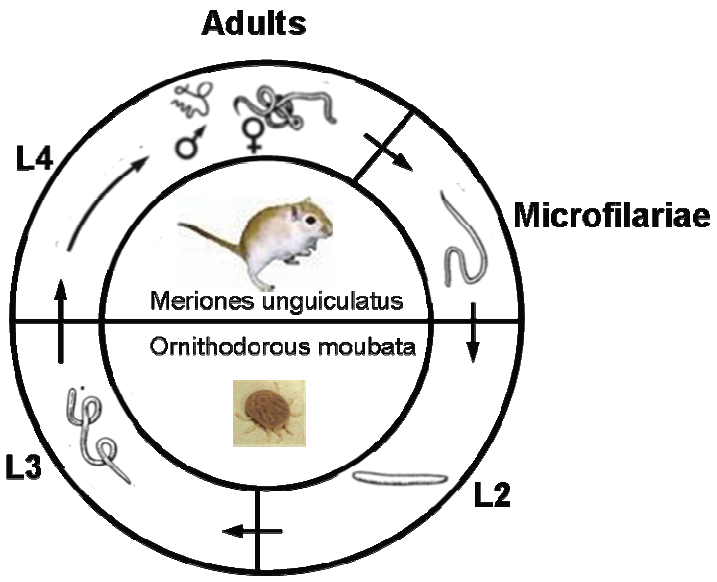


Figure 1. The life cycle of *Acanthocheilonema viteae*. The microfilariae are taken up by the arthropod host (tick – *Ornithodoros moubata*) during the blood meal and develop into the L2 and L3 stages. The infective L3 are transmitted to the rodent host where after 6 weeks of development and migration they settle usually under the skin and develop to adults. Source: Department of Molecular Parasitology.

1.4. Immunological aspects of Helminth infections

Infections with nematodes and other helminths are regularly associated with T-helper cell 2 (Th2) responses, eosinophilia and high levels of IgE, symptoms that are usually found in allergic patients. In the context of helminth infections, IgE and eosinophils can efficiently attack certain helminth stages, e.g. larval stages of schistosomes, by antibody dependent cellular cytotoxicity (Capron and Capron, 1994, see Fig. 2). However, certain immune effector mechanisms are modulated during infection, an effect probably owing to helminth immunomodulators acting on the host. Thus, although nematode infections polarize immune responses towards Th2, which would theoretically favor atopic reactions, they additionally trigger powerful anti-inflammatory mechanisms limiting the magnitude of the *in vivo*-response to allergens. The resulting immunological phenotype has been termed “anti-allergic phenotype”.

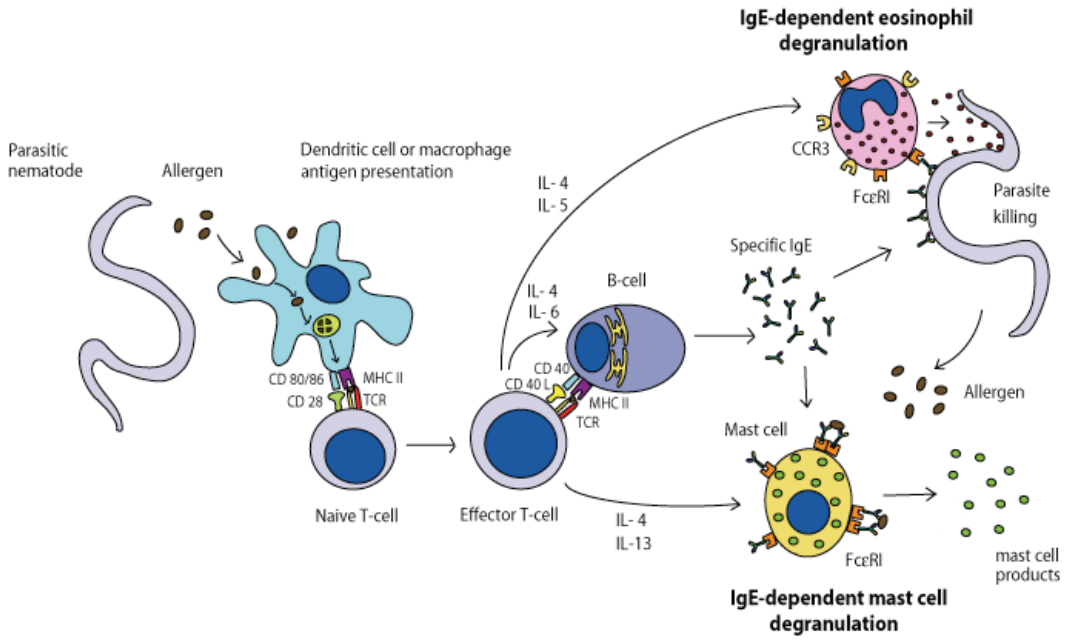


Figure 2. Induction of IgE-mediated anti-helminth effector reactions. Parasitic nematodes release allergens that are presented to naïve T cells by dendritic cells, macrophages or B cells. Activated T cells (effector T cells) release cytokines that activate other cells of the immune system and among others induce production of allergen-specific IgE by B cells. Allergens released by the nematodes can cross-link specific IgE on the mast cell surface and initiate their degranulation. Specific IgE bound to the surface of the parasites can be recognized by eosinophils and initiate their degranulation, eventually leading to parasite killing and tissue damage. CCR3, CC chemokine receptor 3; TCR, T cell receptor; MHC II, major histocompatiblity complex II; FcεR I, receptor I for Fc part of IgE; CD80/86, CD28, CD40, CD40L: costimulatory molecules.

Nematode infections will continuously confront the host with a large number of different antigens. This is particularly evident in the case of filarial infections, where worms living within the host's tissues reportedly reach life-spans of more than a decade. These worms constantly release large amounts of microfilariae that eventually die and degrade after having lived for several months. It was reported that patients heavily infected with *O. volvulus* might harbor about 100,000,000 microfilariae in addition to their adult worms (Schulz-Key and Karam, 1986). This number highlights the enormous amount of foreign material a patient's immune system must deal with.

Out of the many nematode antigens a host is exposed to, some are allergens, meaning that they induce IgE antibodies, which can trigger allergic responses. Such allergic responses consist of the degranulation of basophils or mast cells, when their surface bound IgEs are cross-linked by allergen (Fig. 2). Release of histamine and other mediators, subsequent attraction and activation of effector cells lead to inflammatory responses that may kill the parasite, but can also result in damage of the host tissue.

1.5. Tropomyosin

Tropomyosin was characterized as one of important antigens in the filariae. Studies in *A. vitae* model showed that antibodies derived from serum of animals immunized with irradiated L3 and resistant to the following challenge, when used in Western Blot on whole parasite extract, among others, bind strongly to the 41 kDa band on the whole worm extract. Protein in this band was characterized as the filarial tropomyosin (Hartmann et al., 1997) thus, *A. viteae* tropomyosin seemed to be an immunodominant antigen and a promising potential vaccine candidate. Moreover, invertebrate tropomyosins were described to account for allergic reactions against seafood, cockroaches and house dust mites (Reese et al., 1999). As reported tropomyosin also induces reactive IgE in nematode infections (Asturias et al., 2000; Hartmann at al., 2006). It was also observed that tropomyosin is present on the cuticle of *O. volvulus* larvae (Jenkins et al, 1998). All these arguments place tropomyosin as a major antigen in the context of filarial invasion.

So far vaccination with *E. coli*-expressed proteins of filariae has met with difficulties, because the level of protection observed was generally much lower and showed high

variability between experiments than with irradiated larvae (Abraham et al., 2002; Lustigman et al., 2002). Nonetheless, proteins are still considered as a promising alternative, although it is difficult to find a suitable antigen. Due to its immunogenic properties tropomyosin is an interesting candidate for vaccination experiments.

1.6. Structure and function of tropomyosin

Tropomyosin is a microfilament-associated protein present in all eukaryotic cells. It is found in multiple isoforms that are characteristic for specific types of tissue or cells (Perry, 2001). The most common isoform of tropomyosin has a molecular weight of around 32.5 kDa and is abundant in muscle, where it forms a structure characteristic for all helical proteins, a dimer in the form of a coiled-coil rod with two polypeptides swirling one around the other, of approximately 20 Å in diameter and 400 Å in length (Stewart et al., 2001). In thin filaments, tropomyosin molecules associated in a head to tail manner form overlapping chains that span along actin filaments (Fig. 3A). They play a crucial role in the process of thin filament activation by blocking or freeing actin binding sites in the presence of calcium ions and myosin (Vibert et al., 1997). In this respect tropomyosin is essential in the process of muscle work, proper action of the movement apparatus and the basic functionality of filaments within the cytoskeleton. Activation of tropomyosin is calcium dependent and is controlled by a complex of three troponins, which have a great influence on tropomyosin and its flexibility (Solaro and Rarick, 1998).
The coiled-coil conformation of tropomyosin is a result of the architecture of the protein, which is built from heptapeptides (*abcdefg*). This motif is made up of a sequence of amino acids (aa) with a similar chemical nature, resulting in imperfect repeats of short aa sequences (Paulucci et al., 2002, see Fig. 3B). Both, the N- and C-terminus of tropomyosin have conserved aa residues, which are vital for the creation of “head-to-tail overlaps” allowing tropomyosin polymers to extend over the thin muscle filaments (Sousa and Farah, 2002).

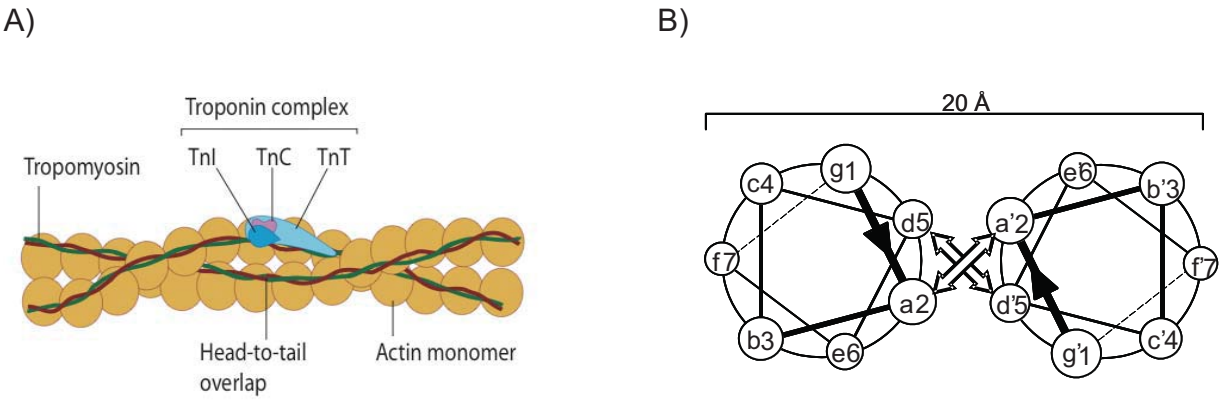


Figure 3. Structure of tropomyosin. Details see text. **A)** Tropomyosin in the context of a thin muscle filament. The tropomyosin multimer is arranged along an actin filament. In the resting stage, tropomyosin covers myosin-binding sites of actin. Ca^{2+} and the proximity of myosin heads lead to activation of troponins (TnI, TnC, TnT) that start a cyclic reaction resulting in a shift of tropomyosin by 35°. This reversible shift exposes the hitherto blocked myosin-binding sites and allows myosin heads to bind, a prerequisite for locomotion (modified after Gordon, 2000). **B)** Structure of the tropomyosin coiled-coil, viewed along the polymer (residues g1 to f7 create a single heptapeptide turn). The close association between two polypeptides results from binding of hydrophobic side chains of aa *a* and *d* of one molecule to aa a' and d' of the partner molecule. Salt bridges between aa *e*, *g* and *g'*, *e'* stabilize the bond. Side chains of aa in positions *b*, *c*, *f*, are generally hydrophilic and face outside where they can react with a residues of adjacent proteins (modified after Stewart, 2001).

1.7. Conserved structure of tropomyosin

The complex interaction with other proteins involved in the process of movement or transport allows only a low level of freedom in aa composition of tropomyosin. Therefore, it is not surprising that the degree of homology is very high among tropomyosins even of phylogenetically distant species. Thus, tropomyosins represent a family of very conserved proteins (Perry, 2001). The homology of aa sequences of tropomyosins originating from invertebrate and vertebrate organisms reaches over 50%. The sequence identity between tropomyosin isoforms of closely related invertebrate species might reach 95% as e.g. between the orthologues of the human pathogenic filarial nematode *O. volvulus* and the rodent filarial *A. viteae* (Hartmann et al., 2006). Detailed studies of a muscle isoform of vertebrate tropomyosin implicated seven regions along the tropomyosin polypeptide that play a key role in interactions with actin. These actin-binding regions are very conserved (e.g. region 5 with 31% of the residues identical throughout the animal kingdom) and typically show a pattern of conserved aa in certain positions within a heptapeptide repeat (Hitchcock-DeGregori et al., 2002).

1.8. Tropomyosin isoforms in nematodes

The free-living nematode *Caenorhabditis elegans* has one tropomyosin gene (*tmy*-1/lev-11) that is located on chromosome I, spans 14.5 kb and includes 14 exons. Alternative splicing of its transcript leads to creation of 4 different tissue-specific isoforms, CeTM I-IV (Kagawa et al., 1995; Anyanful et al., 2001). RNAi-mediated gene silencing in *C. elegans* revealed that tropomyosin is essential for body morphology, coordinated movement and embryogenesis (Anyanful et al., 2001). Considering the high degree of conservation of tropomyosins, it is conceivable that these proteins in parasitic nematodes have similar functions and architecture. Tropomyosin of parasitic worms was found to be abundant in the muscle layer and was shown to occur also in the cuticle of *O. volvulus* (Jenkins et al., 1998) and *Trichinella spiralis* (Nakada et al., 2003).

1.9. Allergenicity of tropomyosin and cross-reactivity among different species

Tropomyosins of invertebrates have been reported to be potent allergens for humans, especially among food allergens (Lehrer et al., 2002). It was established that patients allergic to shrimp are not only allergic to other crustaceans, but also to more distantly related taxa of invertebrates. This cross-reactivity among tropomyosins of different origin was mostly evaluated with competition assays, where binding of serum IgE to its target epitopes was inhibited by addition of tropomyosin from a different source (Tab. 1). It was shown that the cross-reactivity is due to conserved IgE-recognized epitopes shared by closely or distantly related species. Several studies revealed that IgE against tropomyosin of shrimp cross-reacts with tropomyosin of non-edible arthropods such as house dust mites (Witteman et al., 1994; Aki et al., 1995) and insects like cockroaches, grasshoppers and fruit flies (Lehrer et al., 2002). Tropomyosin is therefore considered a cause for widespread cross-reactivity particularly among seafood and aeroallergens of invertebrate origin, suggesting that invertebrate tropomyosins are cross-reactive pan-allergens (Reese et al., 1999).

Table 1. Observed cross-reactivity of human serum IgE with tropomyosins from different edible and nonedible species of invertebrates

Source of tropomyosin	*Cross-reactive agent*	*Reference*
A. simplex (nematode)	house dust mite tropomyosin	(Jeong et al., 2006)
O. volvulus (nematode)	shrimp tropomyosin	(Jenkins et al., 1998)
shrimp	cockroaches, grasshoppers, fruit flies, mites and lobster tropomyosin	(Crespo et al., 1995, Leung et al., 1996, Saarne et al., 2003, Bernardini et al., 2005)
shrimp	house dust mite tropomyosin	(Aki et al., 1995, (Martinez et al., 1997)
lobster	Hom 1 (lobster), Pan s 1 (shrimp), Met s 1 (shrimp)	(Santos et al., 1999)
crab	lobster tropo, shrimp tropo	(Leung et al., 1998a)
silverfish	cockroach, mite and shrimp native tropomyosin	(Asturias et al., 2002)
cockroach	shrimp tropomyosin	(Leung et al., 1998b)
cockroach	other cockroach and mite tropomyosin	(Asturias et al., 1999)
snail	shrimp tropomyosin	(Barletta et al., 2005)

Helminth allergens comprise several other nematode proteins like well characterized polyprotein allergens (NPA). These include ABA-I from *Ascaris suum* or TBA-I from *Toxocara canis*, and also from other intestinal nematodes and filariae (gp15/400 from *B. malayi* or LL20 from *Loa loa*). NPA are lipid-binding proteins with a suspected immunomodulatory function (Kennedy, 2000). Allergens of parasitic nematodes include also the 31 kDa Tco-api-1 protein from *Trichostrongylus colubriformis* (Shaw et al., 2003), the major high molecular weight allergen (HMWtc) from *Teladorsagia circumcincta* (Huntley et al., 2001) and the NIE allergen from *Strongyloides stercoralis* (Ravi et al., 2005). All these proteins evoke strong IgE responses and most induce eosinophilia and Th2 biased cytokine responses as well. Interestingly, to the group of nematode allergens belongs also paramyosin from *Anisakis simplex* (Perez-Perez et al., 2000), a protein that shares many features with tropomyosin.

1.10. Tropomyosin as a vaccine candidate

Nematode tropomyosin is one of the few antigens described to induce significant protective immunity against parasite challenge in vaccination experiments. The existing

data on tropomyosin immunization were brought together in Tab. 2.

Table 2. Vaccine studies with tropomyosin of various parasites

Parasite	***Host***	***Vaccine formulation***	***Reduction in worm burden (%)***	***Comments / approach / reference***
Trichostrongylus colubriformis	Guinea pigs	41 kDa fraction of L3 extract	43-51	(O'Donnell et al., 1989)
Onchocerca volvulus	a)BALB/c mice	C-terminus fragment+MBP (Ov14)	48-62	a) Ov14 was used against *O. lienalis* challenge (Taylor et al., 1996)
	b) Jirds		46 89 (blood mf)	b) Ov14 useds against *A. viteae* challenge (Taylor et al., 1996)
Onchocerca lienalis	BALB/c mice	Aqueous extract of mf immune serum transfer	51 (mf) 54 (mf)	(Folkard et al., 1996)
Onchocerca volvulus	BALB/c mice	Full lenght cDNA	20	i.m. and epidermal (Gene Gun) (Harrison and Bianco, 2000)
Acanthocheilonema viteae	Jirds	a)Native protein	40-64 93 (blood mf)	(Hartmann et al., 1997)
Schistosoma japonicum	BALB/c mice	Native protein	21 30 (liver eggs)	(Cao and Liu, 1998)

These studies describe the protective capacity of filarial tropomyosin as well as tropomyosin of the intestinal nematode *Trichostrongylus colubriformis* and a trematode *Schistosoma japonicum*. However, they did not address the effector mechanisms leading to the protective immunity. Generally, it is believed that filarial nematodes are attacked by mechanisms of antibody dependant cellular cytotoxicity (ADCC). Recent studies in mouse model systems have described IgE responses as pivotal for protective anti-helminth immune responses induced by irradiated larvae (Abraham et al., 2004). Thus, it appears attractive to re-evaluate a role of a surface associated, IgE inducing candidate antigen with respect to potential effector mechanisms in a natural host-parasite-association.

1.11. Aims and objectives of this study

Understanding the physiological functions of proteins required for parasitism could help to develop novel therapeutic and preventive strategies. With this principle in mind the objective of this study was to characterize the molecular and immunological properties of *A. viteae* tropomyosin.

Tropomyosin is an attractive intervention target suitable for vaccine studies. Therefore, it was an aim of this study to investigate its function during infection of *A. viteae* within a rodent and to evaluate the potential of tropomyosin as a protective antigen in an environment of a model that simulates the natural cycle of *O. volvulus*.

In addition, due to described allergenic potential of invertebrate tropomyosin it was important to study the allergenic properties of filarial tropomyosin and other characteristics and physiological functions that could influence the host immune system and the process of infection.

2. Results

2.1. Molecular cloning of A. viteae and O. volvulus tropomyosins

For this study tropomyosin from two filarial nematodes *A. viteae* and *O. volvulus* was used.

A. viteae tropomyosin gene was previously cloned into the pQE30b vector by the group of Dr. S. Hartmann (Hartmann et al., 2006, GeneBank accession number AF000607) within the Department of Molecular Parasitology. That construct was used in this study to transform competent bacteria and produce recombinant *A. viteae* tropomyosin (rAvTropo).

O. volvulus tropomyosin cDNA was isolated from a cDNA library created from female *O. volvulus* RNA by PCR with specific primers. A clone of 855 bp in length was isolated, with a deduced amino acid sequence of 284 residues. The nucleotide sequence of the clone was very similar to that of *A. viteae* tropomyosin and contained an open reading frame extending from the start methionine to a stop codon at position 853-855. Both highly conserved regions, features of tropomyosin (N-terminal region aa 1-8: M-D-X-I-K-K-K-M and the C-terminal aa 240 – 249 L-K-X-A-E-X-R-A-E) were also present in the sequence of the obtained clone. The clone was named *O. volvulus* tropomyosin and inserted into the pET28b+ expression vector. The resulting construct was used to transform competent bacteria and to express and purify the recombinant protein. The theoretical molecular weight of *O. volvulus* tropomyosin (rOvTropo) was 33.2 kDa and the theoretical isoelectric point was pI 4.71. The recombinant protein had an apart molecular weight of 44 kDa on SDS-PAGE.

2.2. Alignment of tropomyosin sequences of A. viteae and O. volvulus with other invertebrates and vertebrates reveals a great level of similarity

Sequence alignment of *A. viteae* tropomyosin with tropomyosin genes of other nematodes showed a high degree of homology. On the amino acid level AvTropo shows 93% identity and (94% similarity) to *O. volvulus* tropomyosin and 87% (92%) identity to *Caenorhabditis elegans* tropomyosin. The homology to chicken cardiac tropomyosin is 52% (70%), to mouse muscle tropomyosin (TM1) 54% (70%) and to human tropomyosin

(TM1) 53% (73%). Homology of *A. viteae* tropomyosin to tropomyosins from other relevant species of invertebrates and vertebrates was summarized in Tab. 3. Amino acid sequences of both filarial tropomyosins used in this study are presented in figure 4 were they were aligned for comparison with sequences of tropomyosins from other species.

```
A. viteae          1 MDAIKKKMQAMKIEKDTALDRADAAKEKVRQMTDKLERIEEELRDTQKKM  50
O. volvulus        1 MDAIKKKMQAMKIEKDNALDRADAAEEKVRQMTEKLERIEEELRDTQKKM  50
P. aztecus         1 MDAIKKKMQAMKLEKDNAMDRADTLEQQNKEANNRAEKSEEEVHNLQKRM  50
H. americanus      1 MDAIKKKMQAMKLEKDNAMDRADTLEQQNKEANIRAEKTEEEIRITHKKM  50
G. gallus          1 MDAIKKKMQMLKLDKENALDRAEQAEADKKAAEERSKQLEDELVALQKKL  50
S. scrofa          1 MDAIKKKMQMLKLDKENALDRADEAEADKKAAEDRSKQLEDELVSLQKKL  50
                     ********* .*..*..*.***.     .    .  . *.*.   .*..
A. viteae         51 MQTENDLNKAQEDLAVANTNLEDKEKKVQKAEAEVPPLNRRMTLLEEELE 100
O. volvulus       51 MQTENDLVKAQEDLSVANTNLEDKEKKVQEAEAEVAALNRRMTLLEEELE 100
P. aztecus        51 QQLENDLDQVQESLLKANIQLVEKDKALSNAEGEVAALNRRIQLLEEDLE 100
H. americanus     51 QQVENELDQVQEQLSLANTKLEEKEKALQNAEGEVAALNRRIQLLEEDLE 100
G. gallus         51 KGTEDELDKYSESLKDAQEKLELADKKATDAESEVASLNRRIQLVEEELD 100
S. scrofa         51 KATEDELDKYSEALKDAQEKLELAEKKATDAEADVASLNRRIQLFEEELD 100
                        * .* .  * *  *.  *   .*    ** .*  ****. * **.*.
A. viteae        101 RAEERLKIATDKLEKATHTADESDRVRKVMENRSFQDEERANTVESQLKE 150
O. volvulus      101 RAEERLKIATDKLEEATHTADESERVRKVMENRSFQDEERANTVESQEKE 150
P. aztecus       101 RSEERLNTATTKLAEASQAADESERMRKVLENRSLSDEERMDALENQLKE 150
H. americanus    101 RSEERLNTATTKLAEASQAADESERMRKVLENRSLSDEERMDALENQLKE 150
G. gallus        101 RAQERLATALQKLEEAEKAADESERGMKVIENRAQKDEEKMEIQEIQLKE 150
S. scrofa        101 RAQERLATALQKLEEAEKAADESERGMKVIESRAQKDEEKMEIQEIQLKE 150
                     *.***  *  **  * ..****.*  **.* *.  ***.    * * **
A. viteae        151 AQLLAEEADRKYDEVARKLAMVEADLERAEERAEAGENKIVELEEELRVV 200
O. volvulus      151 AQLLAEEADRKYDEVARKLAMVEADLERAEERAEAGENEIVELEEELRVV 200
P. aztecus       151 ARFLAEEADRKYDEVARKLAMVEADLERAEERAETGESKIVELEEELRVV 200
H. americanus    151 ARFLAEEADRKYDEVARKLAMVEADLERAEERAETGESKIVELEEELRVV 200
G. gallus        151 AKHIAEEADRKYEEVARKLVIIEGDLERAEERAELSESQVRQLEEQLRIM 200
S. scrofa        151 AKHIAEDADRKYEEVARKLVIIESDLERAEERAELSEGKCAELEEELKTV 200
                     *. .**.*****.****** ..* **********  *    .***.*. .
A. viteae        201 GNNLKFLEVSEEKALQREDSYEEQIRTVSARLKEAETRAEFAERSVQKLQ 250
O. volvulus      201 GNNLKSLEVSEEKALQREDSYQEQIRTVSVRLKEAETRAEFAERSVQKLQ 250
P. aztecus       201 GNNLKSLEVSEEKANQREEAYKEQIKTLTNKLKAAEARAEFAERSVQKLQ 250
H. americanus    201 GNNLKSLEVSEEKANQREEAYKEQIKTLANKLKAAEARAEFAERSVQKLQ 250
G. gallus        201 DQTLKALMAAEDKYSQKEDKYEEEIKVLTDKLKEAETRAEFAERSVTKLE 250
S. scrofa        201 TNNLKSLEAQAEKYSQKEDKYEEEIKVLSDKLKEAETRAEFAERSVTKLE 250
                      ..** *    .*  *.*. * *.*. .. .** **.********* **.
A. viteae        251 KEVDRLEDELVHEKGRYKNISEELDQTFQELFGY* 285
O. volvulus      251 KKVDRLEDELVHEKERYKNISEELDQTFQELSGY* 285
P. aztecus       251 KEVDRLEDELVNEKEKYKSITDELDQTFSELSGY* 285
H. americanus    251 KEVDRLEDELVNEKEKYKSITDELDQTFSELSGY* 285
G. gallus        251 KSIDDLEEKVAHAKEENLNMHQMLDQTLLELNNM* 285
S. scrofa        251 KSIDDLEDELYAQKLKYKAISEELDHALNDMTSI* 285
                     * .* **. .   *     .   **..  ..
```

Figure 4. Alignment of tropomyosin genes sequences of *Acanthocheilonema viteae* and *Onchocerca volvulus* used in this study together with sequences of tropomyosin genes from several major species.

Table 3. Homology and similarity of tropomyosins from several relevant species. Homology shown by upper black number, while blue bottom numbers represent similarity.

	A. vitae	*O. volvulus*	*H. polygyrus*	*C. elegans*	*T. colubriformis*	*T. spiralis*	*T. pseudospiralis*	*E. multiocularis*	*S. mansoni*	*H. americanus*	*P. aztecus*	*P. americana*	*D. melanogaster*	*X. laevis*	*G. gallus*	*M. musculus*	*H. sapiens*
A. vitae	100																
O. volvulus	95 96	100															
H. polygyrus	89 94	93 97	100														
C. elegans	88 92	92 95	94 97	100													
T. colubriformis	89 94	93 97	98 99	95 97	100												
T. spiralis	82 90	87 94	86 93	85 92	86 93	100											
T. pseudospiralis	83 91	87 94	87 94	85 92	87 94	99 99	100										
E. multiocularis	53 73	56 76	56 76	56 75	56 76	59 78	59 78	100									
S. mansoni	55 71	58 74	58 73	58 74	58 74	61 76	61 76	74 88	100								
H. americanus	70 85	74 88	72 87	72 86	72 86	78 89	78 89	61 79	60 76	100							
P. aztecus	68 83	71 85	71 84	70 85	71 85	76 87	76 87	60 80	60 76	92 95	100						
P. americana	65 80	68 83	69 84	68 83	69 84	73 84	73 84	55 76	56 73	82 91	81 89	100					
D. melanogaster	65 79	68 82	68 82	68 82	68 82	72 83	72 83	56 74	56 72	79 88	77 89	83 90	100				
X. laevis	55 71	57 74	58 74	58 74	57 74	58 76	59 77	49 71	50 71	57 74	57 75	54 73	55 72	100			
G. gallus	52 71	55 74	54 73	54 73	54 73	55 75	56 76	45 68	48 69	54 72	54 73	52 72	52 72	82 91	100		
M. musculus	54 70	57 73	56 73	56 72	56 73	57 75	57 75	47 68	48 68	56 72	55 72	53 71	54 71	86 93	90 95	100	
H. sapiens	56 73	58 75	57 74	58 75	57 74	58 76	59 77	49 71	50 71	57 74	55 74	54 74	55 73	94 97	82 91	91 95	100

2.3. Purification of filarial tropomyosins

2.3.1. Recombinant tropomyosin of A. viteae and O. volvulus

Expression of the *A. viteae* cDNA in *E. coli* as a polypeptide fused to a C-terminal sequence of six histidines yielded a protein with an apparent molecular mass of about 42 kDa (rAvTropo) and 44 kDa (rOvTropo) in SDS-PAGE. The recombinant proteins were soluble in aqueous buffers and suitable for purification by nickel chelate affinity chromatography (Fig. 5 and 6). It was possible to purify 3.4 mg of rAvTropo and 4.8 mg of rOvTropo out of a 500 ml bacteria culture, but due to the precipitation during the

process of dialysis the final yield of soluble protein dropped to about 200 µg. Proteins were freed of LPS contaminant by passage through EndoTrap columns and sterilized by filtering through 0.2 µm filters and stored at -20˚C until further use.

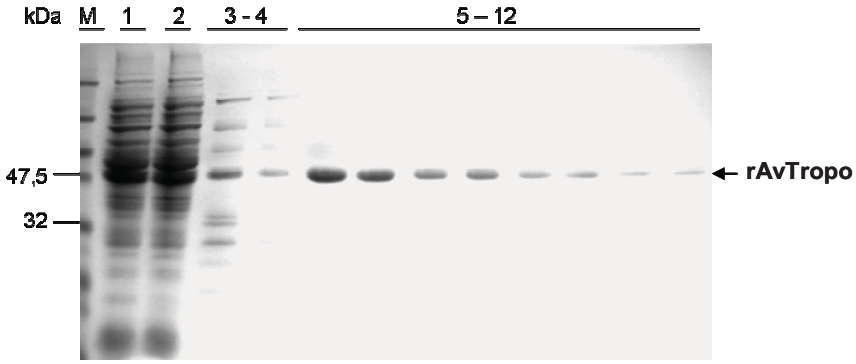


Figure 5. Purification of *Acanthocheilonema viteae* tropomyosin, shown by SDS-PAGE. The single band at the level of 42 KDa in the elution fractions was the recombinant protein. M – Prestained Marker; 1 – Bacterial lysate before Ni-NTA matrix binding; 2 – Lysate flow through; 3-4 – Washing steps; 5-12 – Elution fractions.

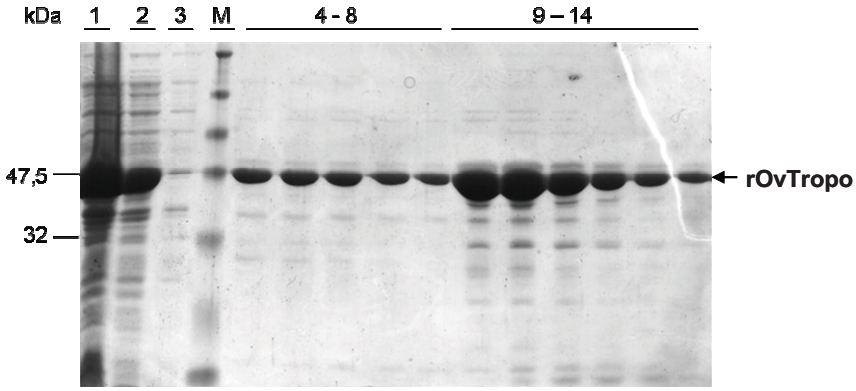


Figure 6. Purification of *Onchocerca volvulus* tropomyosin, shown by SDS-PAGE. Single band at the level of 44 KDa in the elution fractions was the recombinant protein. 1 – Bacterial lysate after induction with IPTG; 2 – bacterial lysate befor binding to NiNTA matrix on Akta; 3 – bacterial lysate flow through; M – marker Prestained; 4-8 – Wash fractions; 9-14 – Elution fractions.

2.3.2. Purification of tropomyosin by electroelution from SDS-PAGE gel

One of the methods to obtain native *A. viteae* tropomyosin utilized in this work was electroelution of the protein band from the SDS-PAGE gel. PBS extract of female worms was first subjected to SDS-PAGE and a band corresponding to tropomyosin (as shown by mAb NR1 in Western-Blot) was cut out from the gel. Tropomyosin was electro-eluted from the gel (eAvTropo) and its quality and purity was checked on another SDS-gel where it appeared as a single band. It was later dialyzed in PBS. It was possible to

obtain about 400 µg of eAvTropo from an exctract of 150 worms. Protein was later filtered through the 0.2 µl mesh to sterilize and frozen at -20˚C until use.

2.3.3. Purification of native A. viteae tropomyosin by affinity chromatography with monoclonal antibody

Native tropomyosin from *A. viteae* (nAvTropo) was purified using monoclonal antibody NR1 bound to CNBr-activated Sepharose 4B. Tropomyosin was purified by the affinity chromatography from the female worms PBS extract. It was possible to purify about 200 µg of pure nAvTropo out of 400 adult female worms (Fig. 7). Protein was purified on EndoTrap column to diminish LPS presence and dialyzed against PBS, sterilized through 0.2 µl filter and stored at -20˚C until further use.

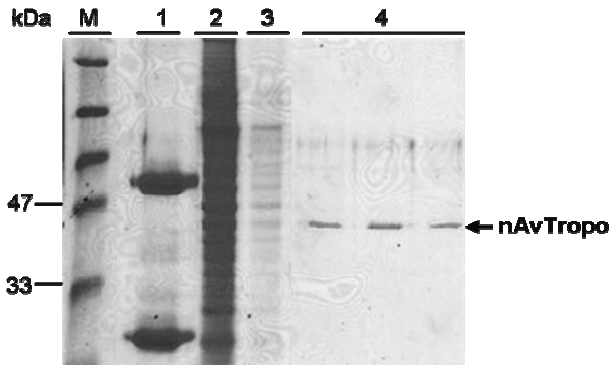


Figure 7. Purification of *Acanthocheilonema viteae* native tropomyosin (nAvTropo), shown by SDS-PAGE. Single band at the level of 38 kDa in elution fractions corresponds to native protein. M – marker Prestained; 1 – mAb NR1 bound on sepharose; 2 – Female *A. viteae* PBS extract; 3 – Wash fraction; 4 – Elution fractions.

2.3.4. Both eAvTropo and rAvTropo retain the secondary structure of an α-helical protein

Purified eAvTropo and rAvTropo were a subject of a Circular Dichroism (CD) spectroscopy analysis. Both forms of *A. viteae* tropomyosin were compared with native and recombinant forms of well characterized shrimp tropomyosin. Analysis revealed that both eAvTropo and rAvTropo molecules have structures resembling α-helical proteins (Fig. 8). Spectra obtained for these proteins were similar to that obtained for proteins from shrimp (recombinant *Penaeus aztecus* tropomyosins - rPen a 1 and native *P. aztecus* tropomyosin - nPen a 1). The shape of spectra for rAvTropo, but not for

eAvTropo was consistent with these of α-helical proteins with characteristic minima at approximately 208 and 222 nm. Predicted secondary structure estimated that rAvTropo had 50.3% α-helical structure, 18.7% β-sheet and 31% random-coiled structure. Similar analysis for eAvTropo showed 22.4% α-helical structure, 44.8% β-sheet, 30.4% random-coiled structure and 2.4% for turn structure. In comparison estimated structures for rPen a 1 were 66% α-helical structure, 2.3% β-sheet, 31,7% random-coiled and for nPen a 1 67%, 2.2% and 30.8%, respectively (Reese et al., 2006). Results obtained for rAvTropo showed that it folded properly and resembled the secondary structure of native tropomyosin as its characteristic were similar to the standard proteins while characteristics of eAvTropo showed that the protein had a different conformation, as the level of α-helical and β-sheet structures differed. Perhaps the procedure of eAvTropo purification altered its natural secondary structure.

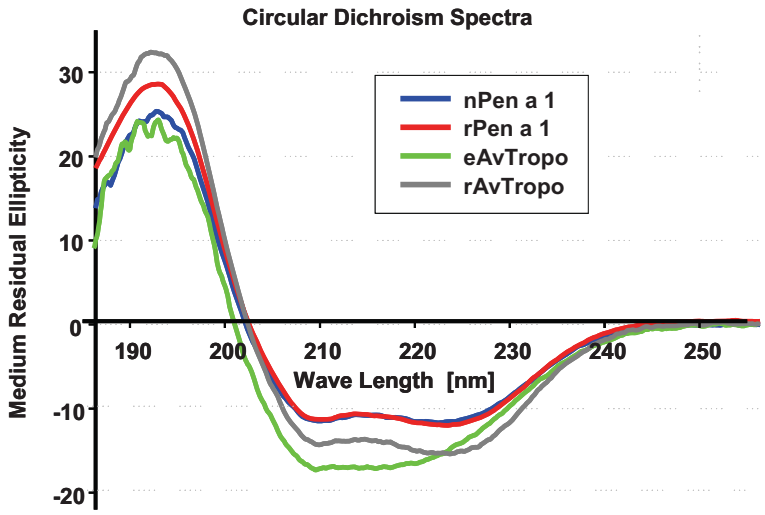


Figure 8. CD spectra of natural and recombinant *Acantocheilonema viteae* tropomyosins in comparison with shrimp tropomyosins (figure prepared in the laboratory of Dr. G. Reese).

2.4. Transfected COS7 cells express A. viteae tropomyosin after 48 h

To analyze the expression of tropomyosin after delivery as a cDNA mammalian cells were transfected *in vitro*. The full length cDNA sequence of *A. viteae* tropomyosin was cloned into the mammalian expression vector pcDNA 3.1+. For expression in eukaryotic cells the construct was transfected into COS 7 cells using the lipid based transfection reagent FuGENE 6. Cells were harvested after 48 hours and transcription of

tropomyosin was checked by RT-PCR with specific *A. viteae* tropomyosin primers. The expression of the protein was confirmed by a Western-Blot with the anti-*A. viteae* tropomyosin mAb NR1.

Transfection of COS7 cells with pcDNA/AvTropo yielded a specific transcript of 860 bp as shown by RT-PCR with specific AvTropo primers, while transfection with the control plasmid did not (Fig. 9A). In immunoblots, mAb (NR1) directed against a specific epitope of invertebrate tropomyosin stained a 38 kDa band present in lysates of COS7 cells transfected with pcDNA/AvTropo. The band was not observed in lysates of untransfected cells or cells transfected with a control plasmid (Fig. 9B). This experiment showed that the DNA construct worked properly and *A. viteae* tropomyosin expression was possible in mammalian cell line. These vital observations allowed the use of this construct in the vaccination experiments.

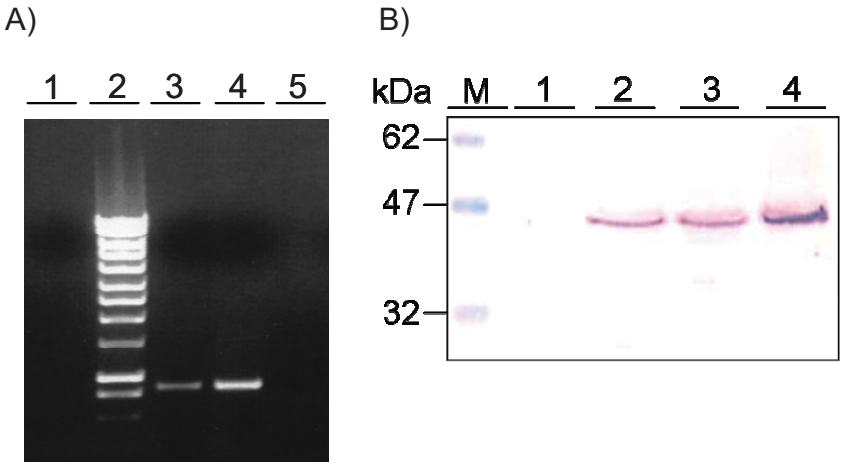


Figure 9. Transfection of COS7 cells with pCDNA/AvTropo construct. **A)** RT-PCR with mRNA isolated from transfected COS7 cells: 1 - negative control for RT-PCR (water), 2 - DNA marker, 3 - transfected cells (1 µg of plasmid DNA), 4 - transfected cells (3 µg of plasmid DNA), 5 - untransfected cells. **B)** Western blot with monoclonal antibody NR1 against *A. vitae* tropomyosin: 1 - untransfected cells, 2-4 - cells transfected with pCDNA/AvTropo 1, 2 and 5 x 10^6 cells, respectively.

2.5. Use of tropomyosin based vaccine against A. viteae challenge infections

In this work several approaches of vaccination with *A. viteae* tropomyosin were utilized. Both, recombinant *E. coli*-expressed full length *A. viteae* tropomyosin and electroeluted worm derived tropomyosin were used as well as a construct with cDNA of the tropomyosin gene. Later a synthetic peptide derived from the tropomyosin sequence was also used. Vaccines in all these approaches utilized various adjuvants like alum, STP or

AdjuPhos. In experiments with proteins and peptides jirds were immunized three times subcutaneously, while all doses of DNA were delivered intramuscularly. Two weeks after a final vaccination experimental jirds were challenged with 70 L3 of *A. viteae*. The challenged L3 were allowed to develop for about 12 weeks after which animals were sacrificed and dissections took place. The read out of the experiments was the number of the adult worms that were recovered from the vaccinated jirds, compared with the respective number in control animals.

2.5.1. Recombinant protein vaccine efficacy depends on adjuvant used

To study the effect and the immunological context of immunization with different tropomyosins, jirds (*Meriones unguiculatus*) were vaccinated with rAvTropo together with either alum or STP as adjuvants. After 11 weeks of experimental infection the animals were sacrificed and the adult worm burdens were determined.

Immunization with rAvTropo induced between 4.2 and 27.7% of protection as evidenced by reduction of adult worm burdens (Tab. 4). The lowest efficacy was observed when tropomyosin was applied together with alum, an adjuvant that promotes Th2 type immune responses (4.2%). Higher protection was observed in groups of jirds immunized with tropomyosin together with STP (Squaleen, Tween, Pleuronic) (27.7%, $p < 0.05$), an adjuvant that strongly induces the Th1 arm of the immune system. Both approaches with recombinant protein as a vaccine did not result in the reduction of the number of circulating microfilariae.

Table 4. Results of immunization with recombinant *Acanthocheilonema viteae* tropomyosin

Antigens used	*Animals no.*	*Surviving animals no.*	*total worm burden*	*Females length (cm)*	*Males length (cm)*	*mf / 20µl of blood at dissection*	*Protection (%) worms reduction*	*Protection (%) Mf reduction*
rAvTropo + alum	**7**	**6**	**20.5 ± 2.7**	**4.59 ± 0.2**	**2.07 ± 0.2**	**465 ± 176**	**4.2 ± 8.6**	**-**
rAvTropo + STP	**7**	**6**	**17.7 ± 7.2**	**4.45 ± 0.3**	**2.1 ± 0.2**	**305 ± 201**	**27.7 ± 27.8 (p<0.05)**	**-**
alum	**7**	**7**	**21.4 ± 5.0**	**4.5 ± 0.4**	**2.21 ± 0.1**	**265 ± 100**	**-**	**-**
STP	**7**	**4**	**24.5 ± 4.7**	**4.72 ± 0.5**	**2.19 ± 0.1**	**220 ± 117**	**-**	**-**

In additional experiments rAvTropo was maleylated before using it for vaccination (malAvTropo). The procedure aimed at preparation of protein that would be targeted to

scavenger receptors on antigen presenting cells (APCs) like dendritic cells or macrophages, to possibly enhance the scale of the antigen uptake and presentation to naive T cells. Animals immunized with malAvTropo showed between 13.7 (with STP) and 22.9% (with FCA) protection (Tab. 5). The results obtained for FCA group were calculated using STP control group.

Immunization with malAvTropo had also moderate influence on the level of microfilariae in the blood of experimental animals. Jirds vaccinated with malAvTropo with STP had on average 18.2% less microfilariae while those immunized with malAvTropo with FCA had 35.8% less microfilariae ($p < 0.05$).

Table 5. Results of immunization with maleylated recombinant *Acanthocheilonema viteae* tropomyosin

Antigens used	*Animals no.*	*Surviving animals no.*	*total worm burden*	*Females length (cm)*	*Males length (cm)*	*mf / 20µl of blood at dissection*	*Protection (%) worms reduction*	*Protection (%) Mf reduction*
malAvTropo + STP	**7**	**7**	**21.4 ± 10**	**4.9 ± 0.4**	**2.26 ± 0.2**	**333 ± 283**	**13.7 ± 8.6**	**18.2 ± 7.9**
malAvTropo + FCA	**7**	**7**	**19.1 ± 9.7**	**4.96 ± 0.5**	**2.36 ± 0.3**	**261 ± 173**	**22.9 ± 23.4**	**35.8 ± 18.6 (p<0.05)**
STP	**7**	**5**	**24.8 ± 4.8**	**5.1 ± 0.4**	**2.28 ± 0.1**	**407 ± 218**	**-**	**-**

Observed results of vaccination with various forms of *E. coli*-derived tropomyosin of *A. viteae* (rAvTropo and malAvTropo) varied between experiments, yet it seems that the protection against challenge with L3 was dependent of the adjuvant used. Animals immunized with vaccine containing adjuvant promoting Th2 type responses (alum) had minimum protection against parasites, while two groups of these vaccinated together with adjuvants inducing a mixed (STP) or only Th1 (FCA) types of immune responses were able to develop partial protection (rAvTropo + alum and malAvTropo + FCA).

2.5.2. Vaccination with worm derived A. viteae tropomyosin

The results of vaccination with rAvTropo suggested that the efficacy of a rAvTropo vaccine relies on the adjuvant used and might depend on the immunological environment and switching towards the protective Th1 type of immune responses. To test whether worm derived *A. viteae*-tropomyosin (eAvTropo) has a better protective capacity than *E. coli*-expressed recombinant protein (owing to the possible

posttranslational modifications or presence of different structural epitopes) we immunized groups of jirds with eAvTropo together with STP.

eAvTropo had a similar efficacy as the best vaccine with the rAvTropo and resulted in 29.4% reduction of adult worm burdens ($p < 0.05$). Immunization with the electro-eluted protein did not result in reduction of circulating microfilariae level in the blood of experimental animals (Tab. 6). These results show that the eAvTropo was not better that the recombinant protein in evoking protective mechanisms in vaccinated animals. Similar protection rates (27.7% for rAvTropo + STP and 29.4% for eAvTropo + STP) indicated that possible posttranslational modifications or other qualities of the worm derived protein did not increase the overall protective effect of a vaccine.

Table 6. Results of immunization with worm derived *Acanthocheilonema viteae* tropomyosin

Antigens used	*Animals no.*	*Surviving animals no.*	*total worm burden*	*Females length (cm)*	*Males length (cm)*	*mf / 20µl of blood at dissection*	*Protection (%) worms reduction*	*Protection (%) Mf reduction*
eAvTropo + STP	**7**	**6**	**17.3 ± 4.9**	**4.58 ± 0.2**	**2.17 ± 0.2**	**359 ± 128**	**29.4 ± 18.6 ($p<0.05$)**	**-**
STP	**7**	**4**	**24.5 ± 4.7**	**4.72 ± 0.5**	**2.19 ± 0.1**	**220 ± 117**	**-**	**-**

2.5.3. Single peptide vaccination

It is unknown if the protective capacity of tropomyosin is conferred by the whole molecule and its structure or only by a limited number of immunogenic sites within that sequence. This led to the idea to immunize a group of jirds with a single synthetic peptide representing an aa sequence of a potent immunogenic site. For this vaccination approach, a peptide comprised of 13 aa was nominated (AQLLAEEADRKYD). This peptide was derived from the amino acid sequence of *A. viteae* tropomyosin and corresponded to the sequence of the *P. aztecus* tropomyosin, described as one of the most potent IgE binding site on the whole molecule (Subba Rao et al., 1999). It consisted both putative T and B cell epitopes.

The synthetic peptide was applied together with STP adjuvant (Tab. 7) and the vaccination resulted in an average protection of 19.3% (not significant) in experimental animals. The level of blood microfilariae was also reduced by 22.3% (not significant).

Table 7. Results of immunization with a single synthetic peptide derived from *Acanthocheilonema viteae* tropomyosin

Antigens used	*Animals no.*	*Surviving animals no.*	*total worm burden*	*Females length (cm)*	*Males length (cm)*	*mf / 20µl of blood at dissection*	*Protection (%) worms reduction*	*Protection (%) Mf reduction*
Av4 + STP	**7**	**5**	**20 ± 11.4**	**4. 8 ± 0.5**	**2.2 ± 0.1**	**316 ± 291**	**19.3 ± 18.6**	**22.3 ± 28.5**
STP	**7**	**5**	**24.8 ± 4.8**	**5.1 ± 0.4**	**2.28 ± 0.1**	**407 ± 218**	**-**	**-**

Protection values in the experiment with a single synthetic peptide (Av4) showed that nominated peptide had no significant effect on L3 challenge. The question if a single immunodominat region can be substituted for a whole molecule in a vaccine and induce similar protective responses has yet to be evaluated.

2.5.4. cDNA based vaccines effects

E. coli-expressed recombinant protein (rAvTropo) and worm derived proteins denatured by SDS-PAGE (eAvTropo) might lack certain conformational epitopes of a natural tropomyosin (as shown by CD spectroscopy). It is therefore plausible that their use in immunization might result in a lower efficacy of the vaccine. Thus, it was important to study the role of native conformation of filarial tropomyosin in the induction of protective immunity. This was achieved by intramuscular injection of jirds with pcDNA/AvTropo, a construct with a *A. viteae* tropomyosin cDNA.

Table 8. Results of immunization with a DNA construct containing *Acanthocheilonema viteae* tropomyosin cDNA

Antigens used	*Animals no.*	*Surviving animals no.*	*total worm burden*	*Females length (cm)*	*Males length (cm)*	*mf / 20µl of blood at dissection*	*Protection (%) worms reduction*	*Protection (%) Mf reduction*
pcDNA/ AvTropo + PBS	**7**	**5**	**27.4 ± 11.8**	**4.80 ± 0.5**	**2.1 ± 0.2**	**551 ± 263**	**18.9 ± 31.1**	**8.6 ± 45.8**
pcDNA + PBS	**11**	**9**	**33.8 ± 9.1**	**5.2 ± 0.9**	**2.4 ± 0.2**	**603 ± 287**	**-**	**-**

Vaccination with pcDNA/AvTropo suspended in PBS led to a reduction of worm burdens by 18.9% (Tab. 8). Differences in adult worms levels were not statistically significant when compared to control animals immunized with empty plasmid. DNA group also showed a non-significant 8.6% reduction in blood microfilariae. Thus, vaccination with a

DNA construct that expresses *A. viteae*-tropomyosin in a supposedly native conformation does not impart stronger protection than vaccination with *E. coli*-expressed or gel-eluted tropomyosin.

When jirds were immunized with pcDNA/AvTropo together with aluminium phosphate as adjuvant, the worm burdens of the animals were reduced by 40.7% ($p < 0.02$) when compared with the animals of the corresponding pcDNA + AdjuPhos control group (Table 9). The observed protection extended to microfilariae, as immunization reduced the burden of worm infectious blood stage larvae by 33.7% ($p < 0.05$).

Table 9. Results of immunization with *Acanthocheilonema* viteae tropomyosin cDNA emulsified in AdjuPhos adjuvant.

Antigens used	*Animals no.*	*Surviving animals no.*	*total worm burden*	*Females length (cm)*	*Males length (cm)*	*mf / 20µl of blood at dissection*	*Protection (%) worms reduction*	*Protection (%) Mf reduction*
pcDNA/ AvTropo + AdjuPhos	**7**	**7**	**16 ± 6.9**	**4.64 ± 0.3**	**2.07 ± 0.2**	**361 ± 167**	**40.7 ± 17.1 (p<0.02)**	**33.7 ± 26.9 (p<0.05)**
pcDNA + AdjuPhos	**10**	**7**	**27 ± 12.7**	**4.68 ± 0.8**	**2.11 ± 0.2**	**545 ± 193**	**-**	**-**

In additional studies the initial cDNA vaccine was boosted with a dose of 25 µg of the recombinant protein in PBS (rAvTropo) injected subcutaneously (Table 10). This DNA-prime protein-boost regime showed higher reduction of the adult *A. viteae* number reaching on average 44.3% ($p < 0.01$). In the same approach 48.7% ($p < 0.02$) reduction of the blood microfilariae was observed.

Table 10. Results of immunization with use of a DNA-prime protein-boost regime with *Acanthocheilonema viteae* tropomyosin cDNA

Antigens used	*Animals no.*	*Surviving animals no.*	*total worm burden*	*Females length (cm)*	*Males length (cm)*	*mf / 20µl of blood at dissection*	*Protection (%) worms reduction*	*Protection (%) Mf reduction*
pcDNA/ AvTropo + rAvTropo	**7**	**6**	**18.8 ± 4.8**	**4.6 ± 0.2**	**2.3 ± 0.2**	**309 ± 132**	**44.3 ± 13.1 (p<0.01)**	**48.7 ± 24.7 (p<0.02)**
pcDNA + PBS	**11**	**9**	**33.8 ± 9.1**	**5.2 ± 0.9**	**2.4 ± 0.2**	**603 ± 287**	**-**	**-**

The results described above showed that the use of a DNA vaccines was a more efficient alternative to the previous approaches based on delivering of both forms of purified tropomyosin. DNA vaccination, especially when adjuvant like AdjuPhos or DNA-prime protein-boost regimes were employed, led to much higher protection values in the experimental groups. It could be explained with the AdjuPhos induced enhancement of

the DNA uptake by myocytes at the site of vaccination, while the DNA-prime protein-boost scheme enabled APCs to recognize and present the antigen to the T cells more effectively. It is also possible that observed results were partially due to the presence of posttranslational modifications on parasite tropomyosin due to production by mammalian cell machinery, thus resulting in better recognition of the protein of parasite origin. However, it remains unclear weather these structures play a role in inducing protective effects.

2.5.5. Effect of vaccination on parasite length and development

Although there were some differences with respect to female and male worms length between different experiments (see Tables above), no statistically significant differences in comparison to control groups were found. As shown in tables the average length of a female *A. viteae* worm varied between 4.4 cm and 4.9 cm and the average length of a male worm reached between 2.1 cm and 2.4 cm. Moreover, no obvious differences in appearance of worms between experimental and control groups were observed. It has to be therefore acknowledged that vaccination schemes used in this study had no visible effect on development of parasites.

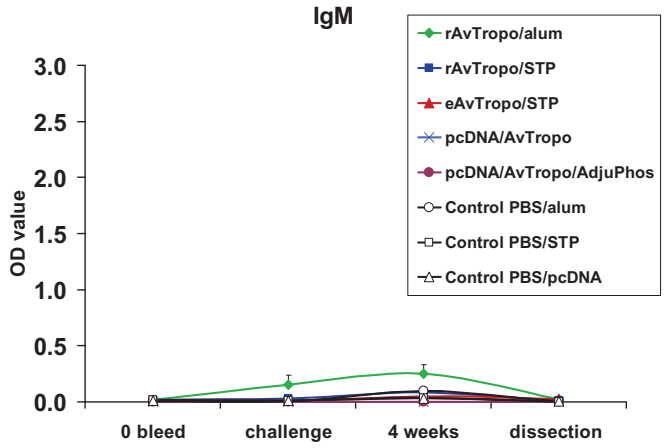
2.6. Analysis of antibody responses of immunized jirds and mice

2.6.1. rAvtropo vaccination elicits IgM and IgG that are not restimulated in the course of infection

The analysis of serum antibodies by ELISA showed that the different modes of immunization induced different patterns of antibody responses against rAvTropo. Application of rAvTropo together with alum resulted in low IgM levels and a strong IgG response at all checked time points in comparison with immunization with rAvTropo and STP ($p < 0.002$). Vaccination together with STP induced very low IgM responses and moderate IgG responses (Fig. 10A, 10B). Interestingly, triple application of eAvTropo induced no detectable antibodies against rAvTropo. Antibody levels were similar to PBS-treated and adjuvant-treated challenge control animals that showed very low antibody responses (Fig. 10A, 10B), although responded well against antigen extract. Application of tropomyosin as a DNA-vaccine resulted in moderate IgG and IgM responses.

Moreover, the DNA vaccine together with AdjuPhos adjuvant failed to evoke strong antibodies responses.

A)



B)

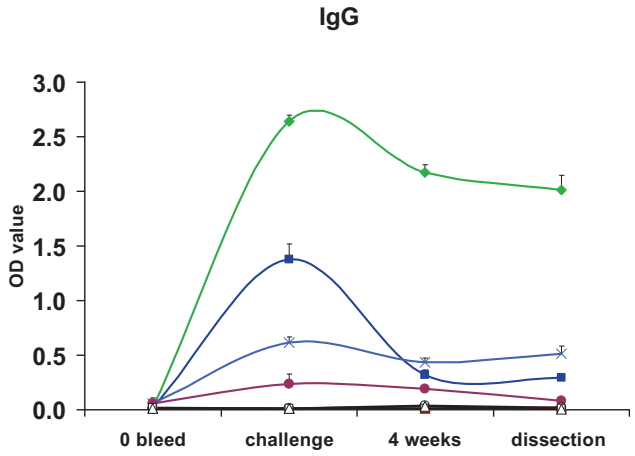


Figure 10. Levels of IgG and IgM antibodies as measured by ELISA in groups of experimental jirds within the period of vaccination trials. rAvTropo was used as a coating antigen. A) Levels of specific IgM antibodies. B) Levels of specific IgG antibodies. Each point shows an average Optical Density value (OD) calculated for 5 to 7 animals.

The observation that jirds immunized with eAvTropo had no detectable antibody responses against rAvTropo indicated distinctive differences in epitope composition or epitope accessibility of the two antigens. In contrast, sera of jirds immunized with rAvTopo recognized eAvTropo tropomyosin suggesting that common epitopes exist. Sera of animals vaccinated with pcDNA/AvTropo contained no IgM, and only marginal amounts of IgG against rAvTropo (Fig. 10A, 10B). The antibody response was even

lower in the group immunized with pcDNA/AvTropo together with aluminium phosphate. Levels of IgG and IgM antibodies in all experimental groups did not increase after challenge infection, suggesting that tropomyosin set free by worms in the context of the infection did not restimulate the IgM and IgG antibody responses induced by the recombinant vaccine. During infection animals of the challenge control group developed little IgM antibodies but failed to raise specific IgG responses against rAvTropo. However, sera of challenge control animals did recognize eAvTropo and reacted with worm extracts in ELISA (not shown). This supports the view that rAvTropo lacks certain antibody-inducing B cell epitopes.

2.6.2. IgG subclasses

An analysis of IgG subclasses revealed that the non-protected animals immunized with rAvTropo/alum had consistently higher titers of IgG1, IgG2a, IgG2b and IgG3 against rAvTropo than the rAvTropo/STP-immunized protected animals (Fig. 11A & 11B). IgG3 was the most abundant subclass in immunized jirds sera, also in group immunized with pcDNA/AvTropo. However, sera of animals from this group had low specific anti-rAvTropo antibody levels (Fig. 11C). The most conspicuous difference between the groups was an almost three times higher IgG1 level in rAvTropo/alum animals as compared to rAvTopo/STP jirds at the time of challege ($p < 0.006$), 4 weeks after infection ($p < 0.02$) and at the point of dissection ($p < 0.02$). Interestingly, like in IgM and total IgG situation, also subclasses of IgG antibodies from sera of naturally infected animals were unable to bind to rAvTropo epitopes (Fig. 11D). It seems that the antibodies generated during natural infection were unable to bind to the epitopes present on rAvTropo. However, when eAvTropo was used as a coating antigen it was bound by IgG (not shown). It is possible that antibodies against the natural tropomyosin epitopes were directed exclusively against structural epitopes that were absent on the recombinant protein.

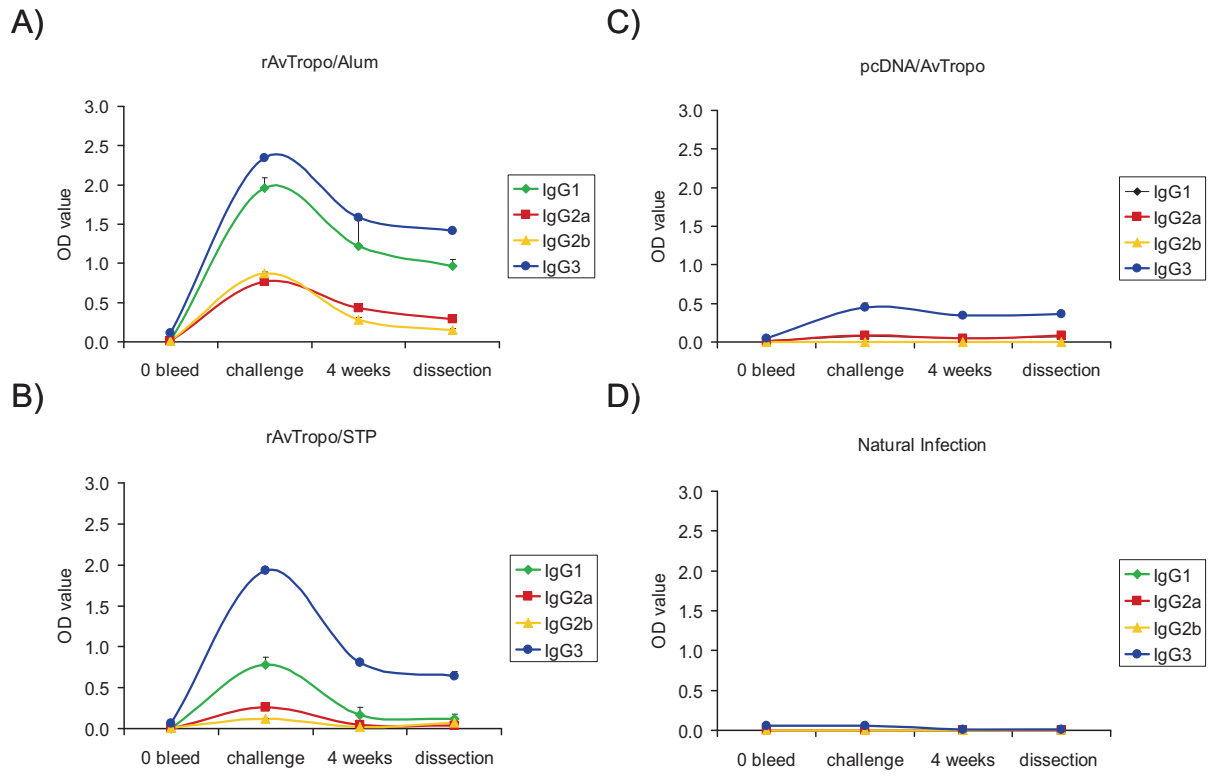


Figure 11. Specific anti-rAvTropo IgG antibody subclasses in sera of vaccinated animals as measured by ELISA in groups of experimental jirds within the period of vaccination trials. rAvTropo was used as a coating antigen. A) rAvTropo/alum immunized group. B) rAvTropo/STP immunized group. C) pcDNA/AvTropo immunized group. D) Sera of animlas from control groups (natural infection). Each point shows an average Optical Density value (OD) calculated for 5 to 7 animals.

The relation between concentrations of IgG1 and IgG2a subclasses can illustrate the changes in vaccination induced dominant T helper response. In rAvTropo/alum relation was 3.3:1, while in rAvTropo/STP and pcDNA/AvTropo it was 2:1 and 1:4, respectively. This shows that in alum approach Th2 way was dominant, but in STP or DNA trials a mixed Th1-biased response was observed.

2.6.3. Immunization with A. viteae tropomyosin induces IgE responses

Interestingly, jirds from groups immunized with recombinant or worm-derived tropomyosin had significantly elevated specific IgE responses before the time point of challenge with *A. viteae* in comparison to control groups ($p < 0.05$, Fig. 12). Unlike IgG, levels of tropomyosin specific IgE rose also after worm invasion, and reached a plateau about 4 weeks pi. In contrast to the situation with IgM or IgG it seems that tropomyosin

released during the course of infection could boost the vaccine-induced IgE response. Sera of animals immunized with rAvTropo/alum and eAvTropo/STP showed highest level of IgE at the point of challenge, while rAvTropo/STP, malAvTropo/alum and pcDNA/AvTropo immunizations did not lead to a significant increase of IgE in comparison to the control groups at the time point of challenge. Moreover, no significant differences in IgE induction between rAvTropo and eAvTropo were observed, suggesting that epitopes for IgE were present on both forms. Interestingly, in contrast to animals immunized with rAvTropo or eAvTropo and control groups, levels of specific IgE in sera of jirds vaccinated with pcDNA/AvTropo and malAvTropo did not rise after the challenge with L3 (Fig. 12).

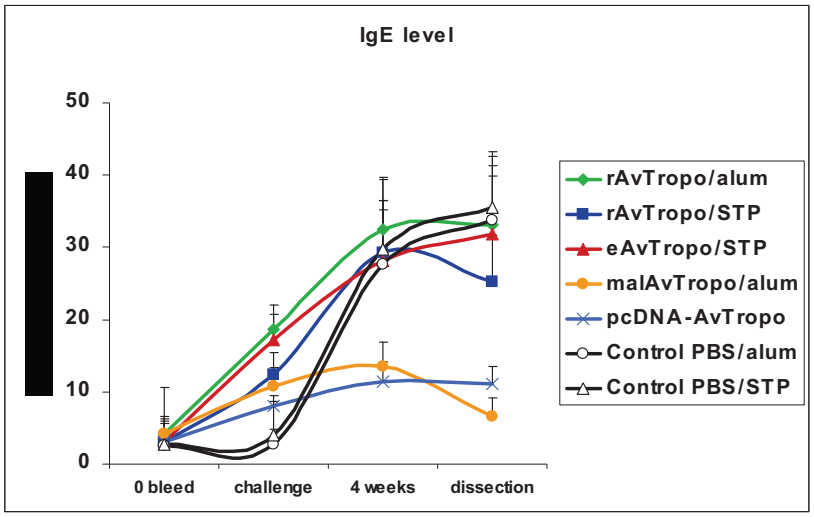


Figure 12. Levels of IgE antibodies as measured with RBL assay in selected groups of experimental jirds within the period of vaccination trials. IgE were cross-linked with 1 µg/ml of corresponding form of tropomyosin. Each point shows an average mediator (β-hexosaminidase) release value in percent, calculated for groups of 5 to 7 animals.

2.6.4. The role of specific anti-tropomyosin antibodies in protecton against L3 challenge

The results described above suggested that antibodies induced by the immunization schemes did not play a major role in assembling protection against filarial L3 challenge. The fact that rAvTropo/STP or pcDNA-immunized animals, which had relatively lower IgG and IgE levels at the time point of infection, were protected, whereas the rAvTropo/alum-immunized animals were not, suggested that the quantity of tropomyosin-

specific IgG and IgE in sera of immunized animals did not correlate with protection against challenge. Indeed it seemed that the higher level of specific anti-tropomyosin antibody in sera of an animal from rAvTropo/alum group could be positively correlated with a greater number of parasites. A similar situation was observed in animals vaccinated with malAvTropo together with STP or pcDNA/AvTropo. Animals from both groups had much lower IgE levels throughout the experiment, having higher protection values (compare Tables 4, 5, and 8). These results indicate that cellular-dependant rather than antibody-dependent mechanisms take part in establishing protection against L3.

2.7. B cell epitopes on A. viteae tropomyosin

2.7.1. Screening of peptide libraries revealed 13 IgG epitopes

To characterize immunodominant B cell epitopes of *A. viteae* tropomyosin we used sera from jirds with natural infection and animals that were vaccinated with rAvTropo + alum or STP to screen synthetic peptide libraries. These were composed of peptides that consisted of 20 or 10 amino acids (20-mers or 10-mers with overlap of 3 aa), representing the whole AvTropo molecule. Libraries were screened with pooled or individual animal sera from infected animals. These approaches resulted firstly (with 20-mers library) in identification of 4 immunodominant regions recognized by IgG antibodies (Fig. 13), which were possible targets for antibody during natural infections. Secondly, a more detailed approach with 10-mere libraries revealed 5 regions recognized by IgG antibodies (Fig. 14 and Tab. 11). Interestingly, IgG from pooled sera of naturally infected animals recognized several recombinant peptides in the 20-mers library, although it did not react with rAvTropo in ELISA (see Fig. 10 and 11).

Figure 13. Analysis of *Acanthocheilonema viteae* tropomyosin IgG epitopes recognized by pooled sera of infected jirds on a 20-mer synthetic peptide library. Dark spots indicate peptides bound by IgG from pooled serum. Arrows point out groups of peptides bound by antibody and circles indicate all peptides relevant for the formation of the antibody binding region.

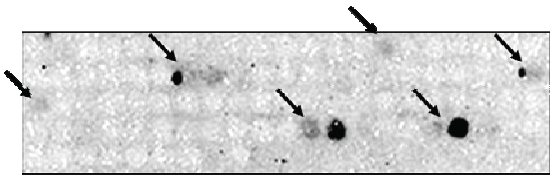


Figure 14. Analysis of IgG epitopes on *Acanthocheilonema viteae* tropomyosin with pooled sera of vaccinated jirds on a 10-mers synthetic peptide library. Pepscan with sera of jirds after three immunizations with rAvTropo + alum. Black spots indicate peptides bound by IgG from serum. Arrows mark relevant peptides.

To characterize AvTropo immunodominant IgG regions in more detail with the aim to characterize actual epitopes additional 10-mer peptide libraries were screened using both pooled and individual sera of BALB/c mice immunized with rAvTropo in PBS or rAvTropo emulsified in alum. The procedure revealed a total of 13 distinct IgG epitopes localized throughout the whole length of the tropomyosin molecule (Fig. 15 and 16). 9 were detectable by pooled sera, whereas 4 more were found when individual sera were used. Individual sera from mice immunized only with rAvTropo in PBS showed a tendency of increased IgG binding to the epitopes localized in the center and the C-terminus of the tropomyosin. Interestingly, N-terminally located epitopes were detectable only in experiment with pooled sera (summarized in Tab. 12) of animals immunized together with alum.

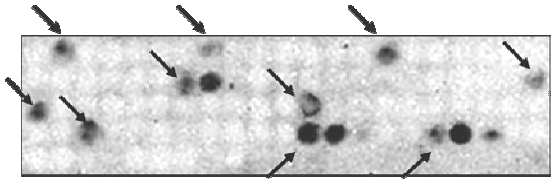


Figure 15. Analysis of IgG epitopes on *Acanthocheilonema viteae* tropomyosins with pooled sera of vaccinated BALB/c mice on a 10-mer synthetic peptide library. Pepscan with sera of animals after three immunizations with 25 µg of rAvTropo + alum. Black spots indicate peptides bound by IgG from serum. Arrows mark positions of bound peptides.

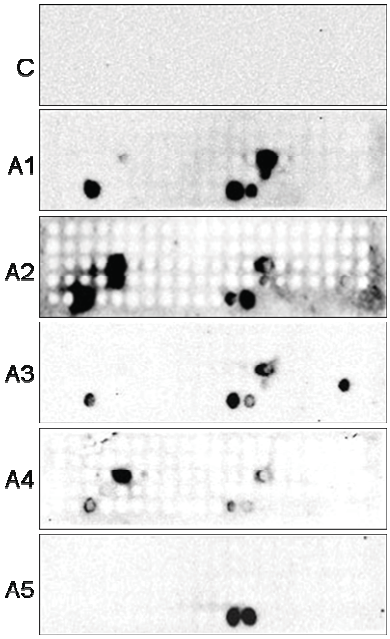


Figure 16. Analysis of IgG epitopes on *Acanthocheilonema viteae* tropomyosin with individual immunized BALB/c mice serum on 10-mers synthetic peptides libraries. Sera after triple vaccination with 25 µg of rAvTropo in PBS were used. Black spots indicate positions of peptides that were bound by serum antibody. C – 0 bleed control, A1-A5 – individual sera reactions with libraries.

The results of analysis with BALB/c sera showed 13 IgG epitopes on *A. viteae* tropomyosin that were spread along the whole tropomyosin molecule. However, it seems that the immunodominant epitopes were localized in the center and on the C-terminus of the molecule, since N-terminaly located ones were detectable only with pooled sera of animals immunized together with an adjuvant that greatly enhances production of specific antibodies. Moreover, a stronger binding of IgG to the epitopes in central and C-terminal regions of the molecule was observed.

2.7.2. 11 IgE epitopes were characterized on A. viteae tropomyosin

A. viteae tropomyosin was shown to be an allergenic antigen that in natural infections induces production of specific functional IgE. To understand the nature of this phenomenon as a first step a characterization of allergenic IgE epitopes on filarial tropomyosin was carried out. Sera of naturally infected jirds depleted of IgG in order to enhance binding of specific IgE were used for the analysis on the 10-mer synthetic peptides libraries. This approach revealed 6 IgE epitopes (Tab. 11) that were recognized by sera of infected animals.

Table 11. Immunodominant B cell epitopes on *Acantoheilonema viteae* tropomyosin in natural infection

A. viteae **IgG** epitopes in natural infection	*A. viteae* **IgE** epitopes in natural infection
LRDTQKKMMQ	NKAQEDLAVA
EAEVPPLNRRMTL	EAEVPPLNRR
DRVRKVMENRSFQ	DRVRKVMENR
RTVSARLKEAETR	NTVESQLKEA
RAEFAERSVQKLQ	EQIRTVSARL
	FEARSVQKLQ

Subsequently, peptide arrays were screened with IgG depleted sera of individual jirds and BALB/c mice immunized with rAvTropo. It allowed further more detailed characterization of IgE binding epitopes (Fig. 17). Results showed that both jirds and mice recognize a very similar pattern of IgE epitopes on filarial tropomyosin. 11 IgE epitopes were characterized. Unlike in the IgG experiment individual sera recognized N-terminally located epitopes showing that immunodominant IgE epitopes are spread throughout the whole tropomyosin molecule. Noteworthy, IgE from jird sera bound a lower number of neighboring peptides, showing perhaps a greater specificity to the epitopes.

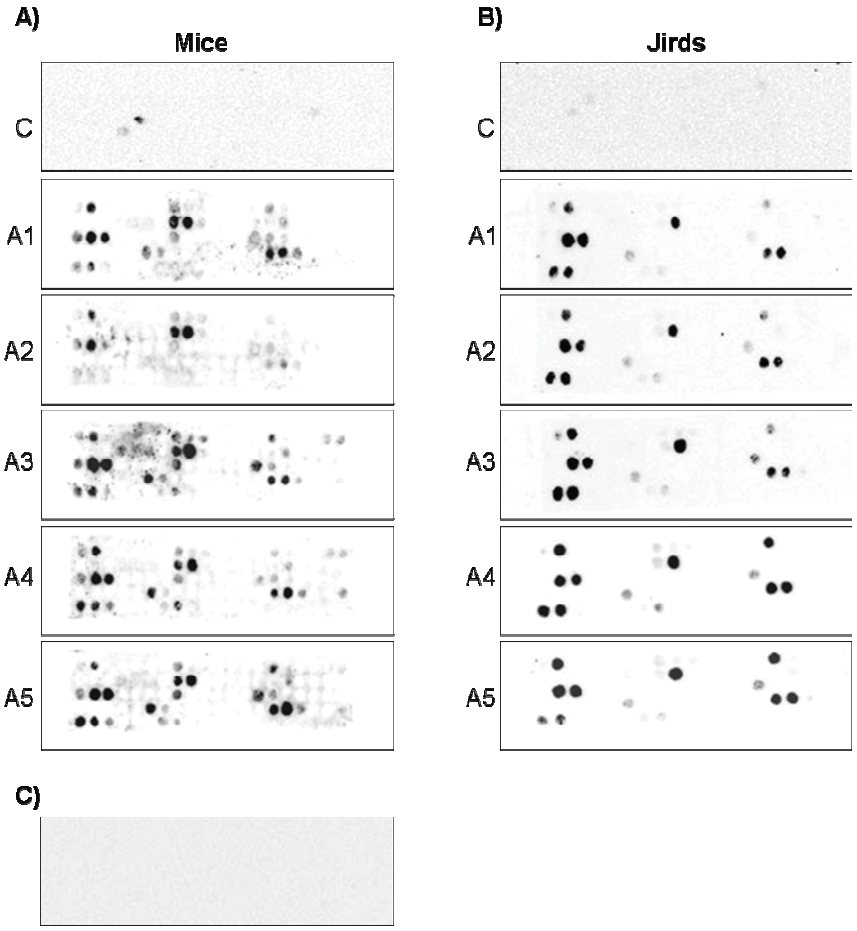


Figure 17. Analysis of IgE epitopes on *Acanthocheilonema viteae* tropomyosins. Sera of single animals were used after triple vaccination with 25 µg of rAvTropo. Sera were depleted of IgG. Black spots indicate positions of peptides bound by IgE antibody. Peptides were characterized in Table 12. **A)** BALB/c mice sera: C – 0 bleed control, A1-A5 single animal sera reactions. **B)** Jirds sera: C – 0 bleed control, A1-A5 single animal sera reactions. **C)** Secondary antibody control.

Like IgG epitopes, IgE specific ones were also distributed along the whole aa sequence of the protein. In fact, out of 11 distinct IgE epitopes identified, 8 epitopes colocated completely or partially with identified IgG epitopes (summarized in Tab. 13). It seems however, that 7 of these epitopes are clustered in 3 distinct IgE binding regions between aa 25 and 67 and aa 106 and 179 and aa 223 and 253 (see Fig. 18). Except mouse 2 serum no major difference in binding intensity of antibodies to the characterized epitopes among individual animal sera was observed. Comparison of both IgG and IgE epitopes positions on *A. viteae* tropomyosin is shown on figure 18.

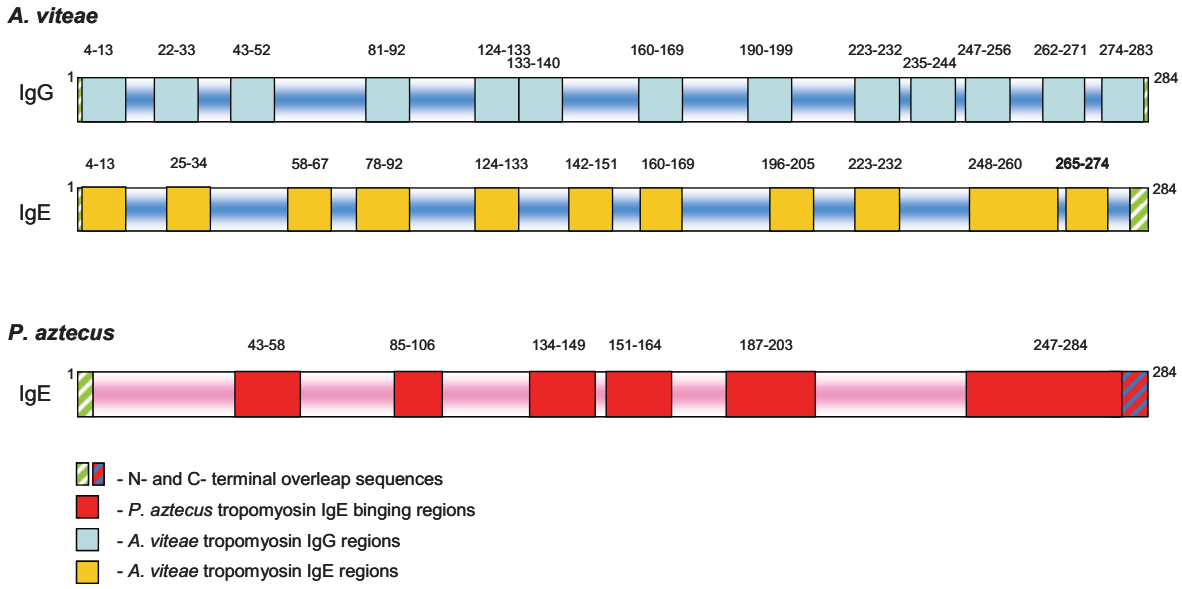


Figure 18. Schematic map of identified *Acanthocheilonema viteae* tropomyosin B-cell epitopes in comparison with identified *Peaneus aztecus* IgE reactive regions taken from Ayuso et al., 2002.

Analysis with BALB/c sera showed a more detailed map of linear B cell epitopes than that obtained from jirds but mirrored the results found in screens with natural infection sera. Interestingly, it can be concluded from these experiments that majority of IgE and IgG epitopes overlap completely or partially. Out of 22 epitopes just 5 (3 for IgG and 2 for IgE) do not share localization for both IgG and IgE binding. Interestingly, positions of most IgE epitopes characterized on *A. viteae* tropomyosin correspond very well with IgE immunodominant regions mapped on shrimp tropomyosin by screening of peptide libraries of this known food allergen with sera from allergic patients (see Fig. 18)

2.7.3. Key amino acid residues within A. viteae tropomyosin IgE epitopes are important for antibody binding

To asses in detail the composition of epitopes found on *A. viteae* tropomyosin and to characterize the role of individual aa residues in the epitope formation experiments with single aa substitutions were undertaken. Major regions containing IgG and IgE epitopes were nominated for the analysis. Each peptide was substituted in subsequent positions with one of 19 other possible amino acids, but only one position at a time was changed. Subsequently, these peptide libraries were screened with the sera obtained from BALB/c

mice after immunization with rAvTropo. For mutagenesis of IgE epitopes, IgG depleted sera were used.

A) IgG B) IgE

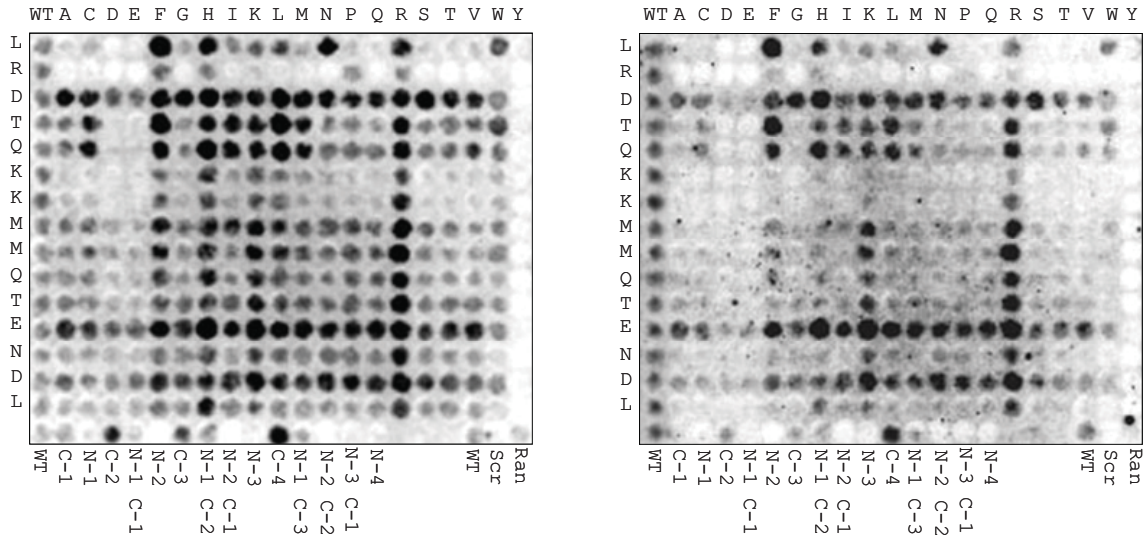


Figure 19. Results of screening of mutated peptide libraries resembling epitope bearing regions with sera of rAvTropo immunized BALB/c mice. **A)** Mutagenesis of IgG binding region pooled serum of 5 animals. Black spots represent peptides bound by IgG. **B)** Analysis of IgE binding to a IgE epitope bearing region. Pooled serum of 5 animals was depleted of IgG and used for analysis. Dark spots indicate positions of peptides that were bound by serum antibody. WT: wild type; Ran: random peptide; Scr: reversed peptide; C-1, N-1 etc.: indicate a number of amino acids deducted from the peptide from C- or N-terminal end. See tables 12 & 13 and figure 20 and 21 for details.

Out of 7 peptides libraries conferring different IgG and IgE epitope regions, 5 were showing reactions suitable for analysis of IgG binding and 3 allowed analysis of IgE binding pattern. The reason for this could be an unnatural folding or an improper length of mutant peptides used to create libraries. However, successful screenings resulted in patterns of spots showing variety of bound peptides. Results showed good reaction with wild type control peptides and no binding to negative controls (inverted and random peptides), indicating that the binding was specific (see Fig. 19 A for example of IgG binding, Fig. 19 B for IgE binding). Figure 20 (IgG) and figure 21 (IgE) represent in a schematic way the substitution results for each of the aa residue that presumably takes part in formation of the linear epitope. Detailed analysis showed several common patterns in recognition of mutant peptides. Most common, substitutions that positively influenced binding of IgG antibody, were with F, H, I, K or R within the epitope, while in

IgE epitopes substitutions with K, L, R or F were promoting binding. These amino acids could replace many other aa in the epitope residues. In parallel, amino acid residues that occurred to be the most important for retaining of the epitope structure and function were R and K, since their replacement in majority of cases led to the abrogation or reduction of the antibody binding.

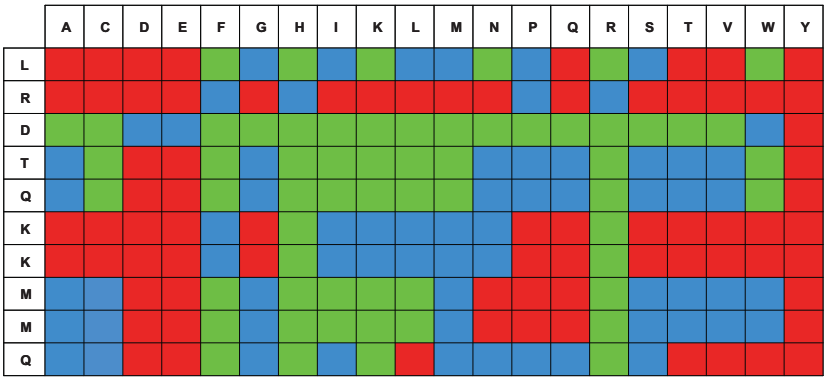


Figure 20. Schematic representation of mutagenesis results of *Acanthocheilonema viteae* tropomyosin IgG epitopes. Synthetic peptides resembling identified epitopes were used for screening with immunized mice sera. Mutant peptides were changed in one amino acid position at a time. Sequence of the peptide used is on the right, amino acid mutation is shown in the top row of the figure. Blue color indicates no change, red color indicates decrease or abrogation of binding, green color indicates enhancement of binding.

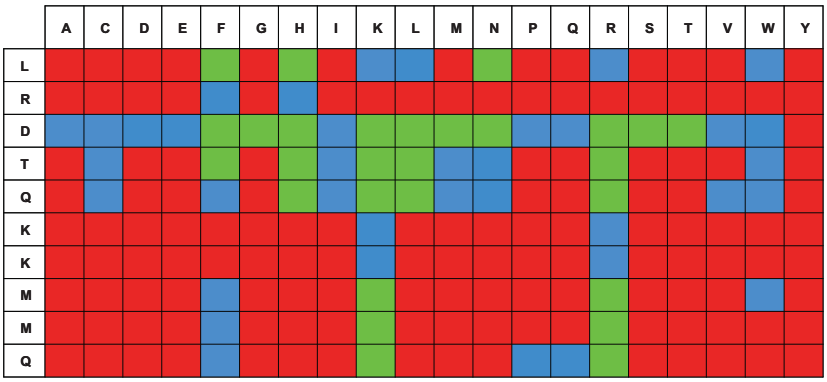


Figure 21. Schematic representation of results of the mutagenesis of *Acantocheilonema viteae* tropomyosin IgE epitopes. Synthetic peptides resembling identified epitopes were used for screening with immunized mice sera. Mutant peptides were changed in one aminoacid position at a time. Sequence of the epitope used is on the right, amino acid mutation is shown in the firs row of the figure. Blue color indicates no change, red color indicates decrease or abrogation of binding, green color indicates enhancement of affinity.

Table 12. Summary table of IgG epitopes containing peptides that were identified on *Acanthocheilonema viteae* tropomyosin

no.	*Epitope sequence*	*Overlap with IgE epitopes*	*Overlap with shrimp IgE epitope*[a]	*Detected by pooled sera*	*Detected by individual sera*	*Remarks*[b]
1	IKKKMQAMKI	yes, M^{1}-I^{13} completely	yes, M^{1}-I^{15} completely	yes	no	recognized weakly
2	ADAAKEKVRQ	no	no	yes	no	recognized weakly
3	LRDTQKKMMQ	yes, L^{44}- Q^{53} completely	yes, V^{43}-D^{56} partially	yes	no	recognized strongly major epitope in natural infection
4	VPPLNRR	yes, V^{81}-R^{86} completely	no	yes	no	recognized strongly major epitope in natural infection
5	RKVMENR	yes, D^{124}-R^{133} completely	yes, R^{132}-Q^{146} partially	yes	no	recognized strongly
6	RSFQDEERAN	yes, D^{125}-R^{134} partially	yes, R^{134}-Q^{148} partially	yes	4/5	recognized strongly major epitope in natural infection
7	RKYDEVARKL	no	no	yes	4/5	recognized strongly major epitope in natural infection
8	ELRVVGNNLK	no	no	yes	4/5	recognized weakly
9	LEEELRVVGN	yes, E^{186}-K^{195} partially	yes, L^{186}-K^{195} partially	yes	2/5	recognized weakly
10	SARL	yes, R^{217}-L^{223} partially	no	yes	4/5	recognized strongly epitope in natural infection
11	RSVQ	yes, F^{232}-Q^{241} partially	yes, K^{239}-Y^{284} partially	yes	4/5	recognized strongly major epitope in natural infection
12	HEKGRYKNIS	no	yes, K^{239}-Y^{284} partially	yes	no	recognized weakly
13	LDQTFQELFG	no	yes, K^{239}-Y^{284} partially	yes	5/5	recognized weakly

[a] – taken from Ayuoso et al., 2002
[b] – for screening of libraries sera from two experiments were used: 1st- pooled sera of 5 immunized mice, 2nd- individual sera of 5 immunized mice

The analysis showed that the amino acids essential for binding in both IgG and IgE case belong to the group of nonpolar hydrophobic (F, I) or basic positively charged (R, K, H) aa. Since lysine and arginine were the critical for binding to the mutant peptides it is possible that their charge and long side chains contributed essentially to the epitope formation. Interestingly, they constitute 33% of all residues within IgG epitopes and 30% within IgE epitopes. Their concentration is also much higher than in the whole molecule where it is 19%.

Table 13. Summary table of IgE epitopes containing peptides that were identified on *Acanthocheilonema viteae* tropomyosin

no.	*Epitope sequence*	*Overlap with IgG epitopes*	*Overlap with shrimp IgE epitope*[a]	*Detected by pooled sera*	*Detected by individual sera*	*Remarks*[b]
1	IKKKMQA	yes, I^{4}-I^{13} completely	yes, M^{1}-I^{15} completely	yes	4/5	recognized strongly
2	KVRQ	no	no	yes	3/5	recognized strongly
3	LRDTQKKMMQ	yes, L^{44}- Q^{53} completely	yes, V^{43}-D^{56} partially	yes	4/5	recognized strongly
4	NKAQEDL	no	no	yes	3/5	recognized weakly present in natural infection
5	LNRR	yes, E^{81}-M^{92} completely	no	yes	5/5	recognized strongly major epitope in natural infection
6	RKVM	yes, D^{124}-Q^{136} completely	yes, R^{132}-Q^{146} partially	yes	4/5	recognized strongly major epitope in natural infection
7	NTVESQLKEA	no	yes, R^{132}-Q^{146} partially	yes	4/5	recognized strongly present in natural infection
8	DEVARKL	no	no	yes	4/5	recognized strongly
9	VVGNNLK	yes, L^{187}-K^{195} completely	yes, E^{177}-G^{191} partially	yes	3/5	recognized strongly
10	SARL	yes, S^{210}-V^{218} partially	no	yes	2/5	recognized weakly epitope in natural infection
11	RSVQ	yes, A^{225}-R^{234} partially Q^{237}-L^{246} partially	yes, K^{238}-Y^{284} partially	yes	4/5	recognized strongly major epitope in natural infection

[a] – taken from Ayuoso et al., 2002
[b] – for screening of libraries sera from two experiments were used: 1st- pooled sera of 5 immunized mice, 2nd- individual sera of 5 immunized animals

2.8. Allergenicity of tropomyosin

A. viteae tropomyosin has a great potential to induce IgE antibodies during a natural infection and after immunization with the recombinant or native protein. Sera of all groups immunized with *A. viteae* tropomyosin consistently contained specific and functional anti-AvTropo IgE antibodies. In contrast to IgG and IgM antibodies, IgE responses against rAvTropo (see Fig. 12) were restimulated by the challenge infection implying that dominant IgE-inducing epitopes were common to both recombinant and worm derived tropomyosin. Later experiments showed that there are at least 11 IgE epitopes on *A. viteae* tropomyosin that contribute to the allergenicity of the protein (Tab. 13). However, it remained to asses the actual potential of rAvTropo and nAvTropo to induce functional IgE and trigger allergic mechanisms. It was also interesting to compare the potential of *A. viteae* tropomyosin with other tropomyosins including those cloned from other parasitic nematodes.

2.8.1. A. viteae tropomyosin is a potent IgE inducing antigen

Both full-length recombinant and native *A. viteae* tropomyosin have strong allergenic potential. The IgE inducing capability of native protein was also well visible during a natural infection of jirds with *A. viteae*, when high level of specific IgE were induced (Fig. 12). To test the specificity of anti-tropomyosin IgE, and to show that induced antibodies were functional multiple RBL assays were performed. Sera from BALB/c mice immunized with eAvTropo or rAvTropo were used to sensitize cells of a rat basophile line and they were stimulated with tropomyosin from different invertebrate and vertebrate species along with control non allergenic proteins.

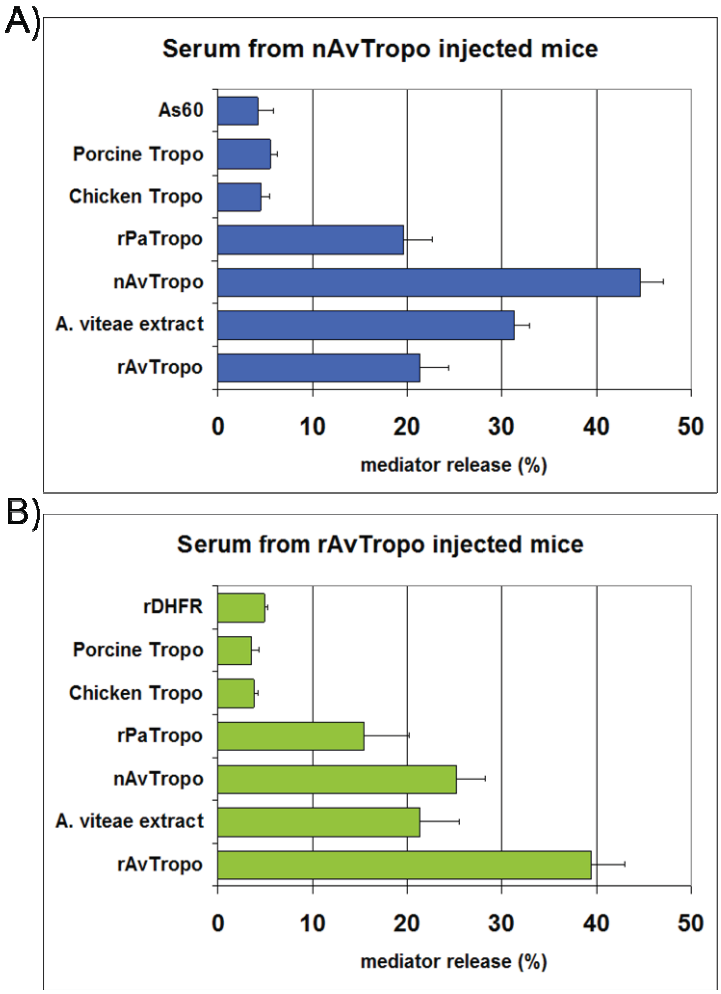


Figure 22. The comparison of allergic potential of *Acantocheilonema viteae* tropomyosin with tropomyosins of other species as shown with RBL assay. A mediator β-hexosaminidase was released by basophiles upon stimulation (%). **A)** Sensitization of cells with pooled serum from mice immunized with nAvTropo in PBS. **B)** Sensitization of cells with serum from mice immunized with rAvTropo in PBS. As60: *Ascaris suum* protein 60 kDa; rPaTropo: recombinant *Peaenus aztecus* tropomyosin; rDHFR: recombinant Dehydrophylase Reductase of rat.

Figure 22 A shows the release of β-hexosaminidase by RBL cells sensitized with sera of mice immunized with nAvTropo. RBL cells upon restimulation with native tropomyosin released almost 45% of their mediator granules into the supernatant. The effect of total worm extract was noteworthy (32%). It is interesting that recombinant tropomyosin from shrimp (rPen a 1) described as a pan-allergen led only to 20% release of mediator, similarly to the value obtained for the recombinant form of AvTropo. Vertebrate tropomyosins from porcine and chicken muscle did not elicit the release of the mediator

from sensitized cells at the comparable levels. The native nematode protein AS60 used as a control also failed to induce the release of mediator.

The degranulation assay with sera of mice immunized with rAvTropo revailed that recombinant, but not native tropomyosin led to the highest release of the mediator (40%, Fig. 22 B). nAvTropo and rPen a 1 led to the release on the level of about 25% and 16%, respectively. Vertebrate tropomyosins and the control protein rDHFR did not evoke release of the mediator at a significant level.

These results suggest differences in IgE epitopes on the surface of worm derived and *E. coli*-expressed tropomyosin, that allowed only partial cross-reactivity. The discrepancy can be attributed to the differences in secondary structure (conformation) of two proteins and perhaps to the presence of posttranslational modifications on eAvTropo that could contribute to the formation of unique epitopes. These suppositions are corroborated by results obtained with total worm extract (Fig. 22 A), where lesser overall tropomyosin concentration led to a higher level of mediator release than for rAvTropo when RBL cell sensitized with sera of nAvTropo immunized mice were used. Although aa sequences of tropomyosin molecules from shrimp and *A. viteae* are similar, and occupy similar localizations as shown by analysis (see Fig. 18 for comparison) epitopes on shrimp tropomyosin were not identical and differed from epitopes on filarial tropomyosin. This resulted in lower degranulation rate when rPen a 1 was used for restimulation of cells. It can be concluded that filarial tropomyosin has a greater allergenic potential in this system than shrimp tropomyosin, as it was able to induce a stronger release of mediator. The difference in release can be also attributed to the more natural folding of the shrimp protein.

2.8.2 Nematode tropomyosins are strongly cross-reactive

To check the cross-reactivity between tropomyosins from various species recombinant proteins from several nematode species and shrimp tropomyosin along with native vertebrate tropomyosin from chicken were used to stimulate sensitized cells in a dose dependent manner (Fig. 23). The assay utilized basophiles sensitized with serum of rAvTropo immunized mice.

The cross-reactivity experiment showed that IgE epitopes on two filarial tropomyosins (*A. viteae* and *O. volvulus*) were very similar and these molecules had similar allergenic potential. Although sera from rAvTropo immunized mice were used, higher levels of mediator were released by cells in presence of rOvTropo (58%) in comparison to for rAvTropo (51%). Surprisingly, recombinant tropomyosins from the parasitic nematode *Heligmosomoides polygyrus* and the free living *C. elegans* induced lower levels of mediator release (33% and 26%, respectively, Fig. 23).

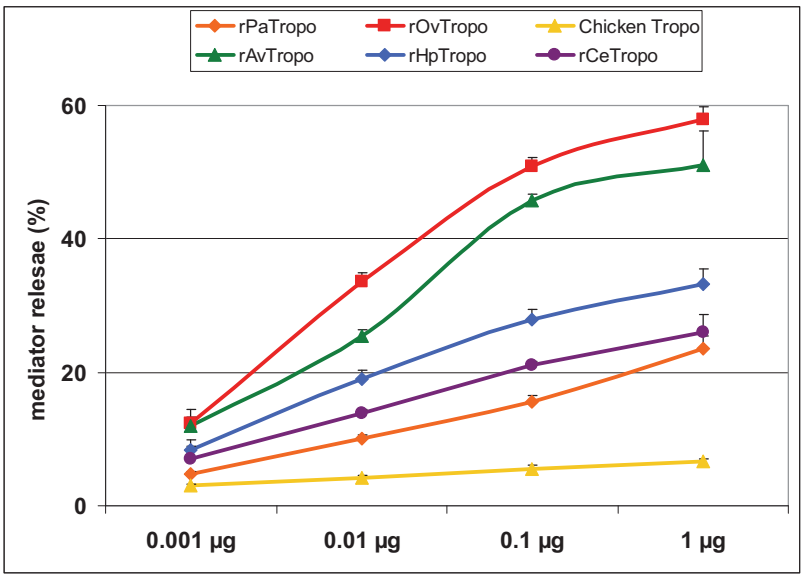


Figure 23. Allergic potential of tropomyosin from different sources as measured by RBL assay. RBL cells were first sensitized with serum of rAvTropo immunized mice and incubated with tropomyosins from various species at several concentrations.

These results show differences between functional IgE epitopes on various nematode tropomyosins. As in previous experiment, tropomyosin from *P. aztecus* did not elicit high levels of degranulation of RBL cells (23%). Chicken tropomyosin used for control evoked only 7% release by the highest dose. Thus, the comparison showed that two filarial tropomyosins were the most cross-reactive. Interestingly, the degree of mediator release was dose dependant. To cross-link enough IgE on the surface of basophils to induce the same mediator release levels as filarial proteins, tropomyosins from other nematodes and shrimp needed to be present in the concentration 10 or even 100 times higher. Example of chicken tropomyosin showed that vertebrate tropomyosin did not cross-react with filarial ones regardless of the concentration.

2.8.3. Determination of the cross-reactivity degree between tropomyosins by competition assay

To dissect the cross-reactivity between various tropomyosins and to substantiate the previous data, competition assays were performed. Experiments relied on depletion of specific anti-*A. viteae* tropomyosin IgE from sera by incubation with tropomyosins from different species. Depleted sera were used to sensitize RBL cells and rAvTropo in several concentrations was used later as a cross-linking antigen.

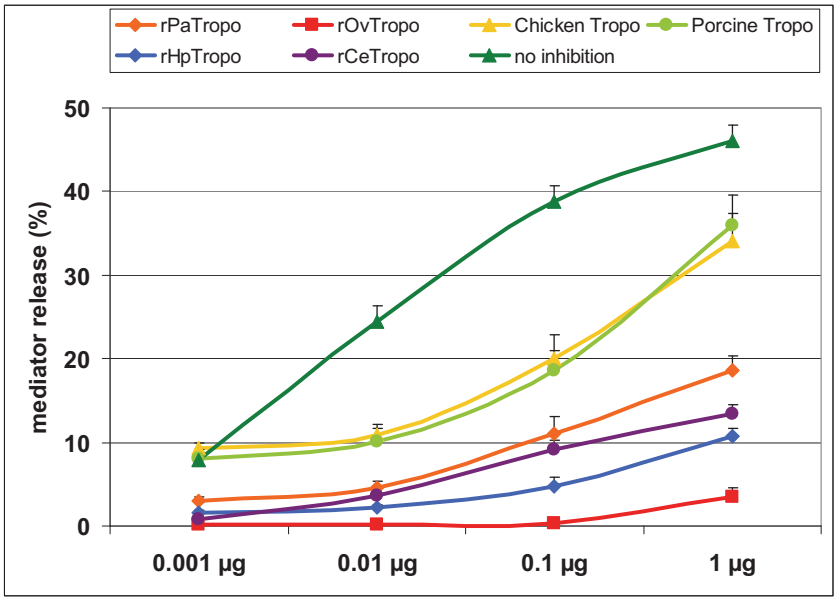


Figure 24. Competition assay using rAvTropo immunized mouse serum and tropomyosins from different species as factors depleting specific IgE, shown by RBL assay. rAvTropo in several concentrations was used as a cross-linking antigen to initiate degranulation.

The competition assay showed that the preabsorbtion of sera led to the much lower sensitization of basophils and mediator release. When rOvTropo was used release from RBL cells was 93% ($p < 0.03$) less as compared to normal release for rAvTropo without preincubation (Fig. 24). Tropomyosins from the *H. polygyrus* and *C. elegans* were also able to reduce the amount of functional IgE in the sera. The reduction of mediator release was 79% and 74%, respectively ($p < 0.05$). Also, preincubation of sera with recombinant tropomyosin from shrimp resulted in 64% ($p < 0.05$) reduction of the mediator release. In contrast, vertebrate tropomyosins (porcine and chicken) were

unable to absorb IgE specific to *A. viteae* in an amount significant enough to inhibit cross-linking by rAvTropo.

The competition among IgE epitopes on invertebrate tropomyosins, but not on vertebrate ones, suggest their structural similarity. Since all tropomyosins have similar α-helical structure, it is plausible that the cross-reactivity of invertebrate tropomyosins is owing to similar IgE epitopes and not to differences in the general structure of the molecules

2.9. Raising monoclonal antibodies against A. viteae tropomyosin

The monoclonal antibody against vertebrate tropomyosins available on the market (TM311), poorly recognize invertebrate forms of the protein. To develop better monoclonal antibodies for affinity purification of native tropomyosin, mice were immunized with recombinant or worm derived tropomyosin of *A. viteae* and their spleen cells were fused with mouse X63.Ag8 myeloma cells. Three out of 90 resulting hybridomas clones that produced antibody against eAvTropo and rAvTropo were chosen for the expansion what resulted in production of mAbs NR1 (IgG1), R21(IgM) and N11 (IgM). These antibodies were used for further studies in both Western Blots and ELISA in parallel with commercially available control antibody against chicken gizzard tropomyosin (TM311, IgG1, Sigma) and the control mAb against *A. viteae* chitinase (clone 24-4, IgG1, Department of Molecular Parasitology).

2.9.1. NR1 but not R21 and N11 mAbs is specific to invertebrate tropomyosins

An immunoblot analysis revealed that mAb NR1 recognized both rAvTropo and an 45 kDa protein band of a native tropomyosin occurring in adult female and male nematodes extracts, as well as in L3 and microfilariae (Fig. 25 A) as well as eAvTropo (not shown). R21 mAb showed similar pattern of recognition but it was also able to bind to the porcine tropomyosin, showing that it is not specific only to invertebrate tropomyosin (Fig. 25 B). N11 antibody was able to detect native tropomyosin but it also bound several other bands within the extract. N11 was unable to recognize rAvTropo and porcine tropomyosin (Fig. 25 C). NR1 and R21 recognized rAvTropo what suggested that target epitopes were created by the aa backbone of the protein and that posttranslational modifications were not playing part in their formation.

We analyzed the binding of these mAbs to extracts from various invertebrate and vertebrate organisms that included also species relevant for human health like parasites or the ones that cause allergies. Table 14 summarizes results and compares with the properties of TM311 and 24-4 control mAb.

Both NR1 and R21 recognized a wide array of native invertebrate tropomyosins (Tab. 14). NR1 bound well to native *A. viteae* tropomyosin, while R21 showed stronger binding to a recombinant form of *A. viteae* tropomyosin. N11 recognized many invertebrate tropomyosins, it also bound to vertebrate tropomyosins from the PBS muscle extracts. TM311 weakly bound to tropomyosin in female and male *A. viteae*, but it could not detect tropomyosin in L3 and Mf. In contrast, it bound to tropomyosin bands from all vertebrate species (both PBS extracts and prepared proteins).

Results showed that NR1 was specific to invertebrate tropomyosins, while N11 and R21 bound also vertebrate proteins from soluble extracts but not prepared by Sigma. All self produced monoclonal antibodies had better recognition of invertebrate tropomyosins than TM311.

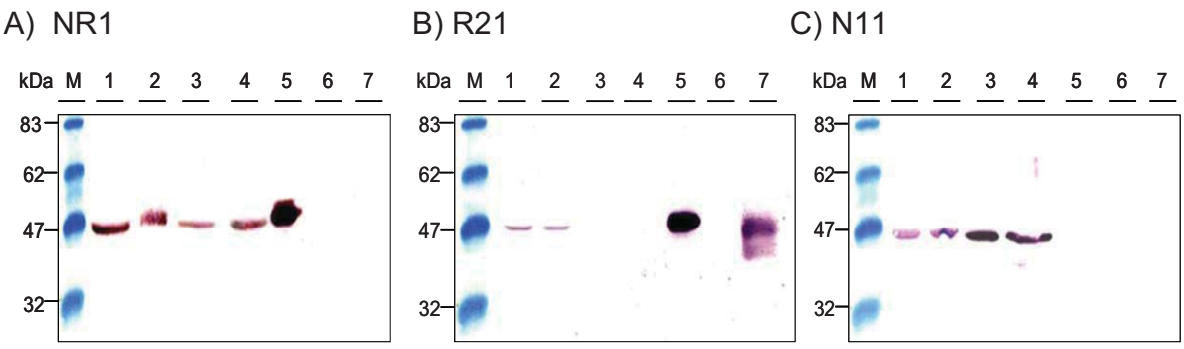


Figure 25. Recognition of *Acanthocheilonema viteae* tropomyosin by monoclonal antibody in Western blot. **A)** NR1, **B)** R21, **C)** N11. M – Prestained marker, 1 – Female worms extract, 2 – Male worms extract, 3 – L3 extract, 4 – microfilariae extract, 5 – rAvTropo, 6 – rPen a1 (shrimp tropomyosin), 7 – porcine tropomyosin.

Tab. 14. Recognition of tropomyosins from different sources by monoclonal antibodies used in this study. (-) – no binding, (+/-) – very week binding, (+,++,+++) – week, moderate, strong binding

Antigen used	*NR1*	*N11*	*R21*	*TM311*	*24-4*
A. viteae female extract	++	++	+	+/-	-
A. viteae male extract	++	++	+	+/-	-
A. viteae L3 extract	++	++	+/-	-	-
A. viteae Mf	++	++	+/-	-	+/-
nAvTropo (afp NR1)	++	++	+	+/-	-
rAvTropo	++	-	+++	+/-	-
O. volvulus total extract	+	++	-	-	-
H. polygyrus total extract	++	+	-	-	-
H. polygyrus L3	+	+	-	-	-
A. suum (uterus) ectract	-	-	-	-	+
A. suum total extract	+	+	-	-	-
C. elegans total extract	++	++	+	+/-	-
E. multilocularis metacestode extract	++	+	+	+/-	-
H. diminuta total extract	++	++	-	-	-
S. mansoni total extract	++	+/-	-	-	-
D. pteronyssinus total extract	-	-	-	+	-
S. scabei total extract	-	-	-	-	-
P. aztecus muscle PBS extract	+	+	-	+	-
B. glabrata total extract	+	+	-	-	-
P. luciperca muscle PBS extract	-	+	-	+	-
G. gallus gizzard tropomyosin (Sigma)	-	-	-	+++	-
G. gallus breast muscle PBS extract	-	+	-	++	-
S. scrofa muscle tropomyosin (Sigma)	-	-	+	+++	-
S. scrofa muscle PBS extract	-	+	-	++	-
M. musculus muscle PBS extract	-	+	-	+++	-
mamalian cells (COS7)	-	-	-	++	-

2.9.2. Epitopes of NR1 and R21 mAb are located in C-terminal part of A. viteae tropomyosin

The target epitopes of mAbs were mapped by screening of overlapping 20-mer and 10-mer peptide libraries representing the aa sequence of *A. viteae*-tropomyosin. Results showed that NR1 and R21 recognized a similar region in the C-terminal end of tropomyosin molecule representing the epitope, while N11 mAb did not bind to the peptides. In addition, TM311 and 24-4 antibodies did not recognize any of the synthetic peptides.

The epitope of NR1 mAb is localized within a stretch of 20 amino acids (Arg^{226}-Ser^{245}) RTVSARLKEAETRAEFAERS (Fig. 26 A), while epitope of mAb R21 is located within the region of 26 amino acids (Val^{253}-Arg^{266}) VDRLEDELVHEKGR (Fig. 26 B). Interestingly, no binding to shorter peptides was observed for either of the antibodies. This suggest

that the epitope conformation was important for binding of mAbs. The possible explanation for the failure of the screening might be that short peptides where unable to adopt correct secondary structures, thus the antibodies could not bind the epitope.

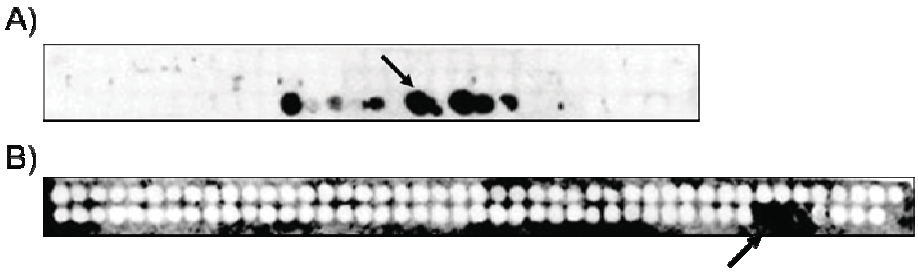


Figure 26. Regions recognized by mAb against tropomyosin on *A. viteae* tropomyosin synthetic peptides (20-mers) libraries. **A)** Screening with NR1 mAb. **B)** Screening with R21 mAb. Black spots indicate peptides bound by IgG from serum. Arrows mark relevant regions.

In order to compare sequences of the target regions of NR1 and R21 available in the data base tropomyosins sequences were aligned. The sequence of NR1 binding region (Arg^{226}-Ser^{245}) was more conserved among tropomyosins (especially LKEAETRAEFAERS sequence seems to be very strongly conserved) that the one where R21 binds (Val^{253}-Arg^{266}). Data obtained after screening of peptide libraries with these two mAb corroborate observations from Western blots that showed different patterns of tropomyosin recognition by these antibodies (see Tab. 14). Additionally, previous epitope analysis with sera of immunized jirds and mice already located functional B cell epitopes in both regions of mAb binding. Interestingly, observed regions are very similar in other invertebrate tropomyosins, but within vertebrate tropomyosins chemical nature of some of their residues differ, explaining lack of mAb binding.

2.9.3. N11 is presumably targeted against posttranslational modification on A. viteae tropomyosin

The inability of N11 to bind any of the peptides in the library of *A. viteae* tropomyosin contrasted with results from Western blot and ELISA showing binding to parasite PBS extract. This suggested that N11 was targeted against posttranslational modifications on a native protein. Also, the presence of additional bands in the blot of *A. viteae* extract suggests an epitope that is partially or entirely created of other than aa moieties. To

check this hypothesis deglycosylated nAvTropo and worm PBS extract were used as coating antigens in ELISA with N11 (Fig. 27 A and B).

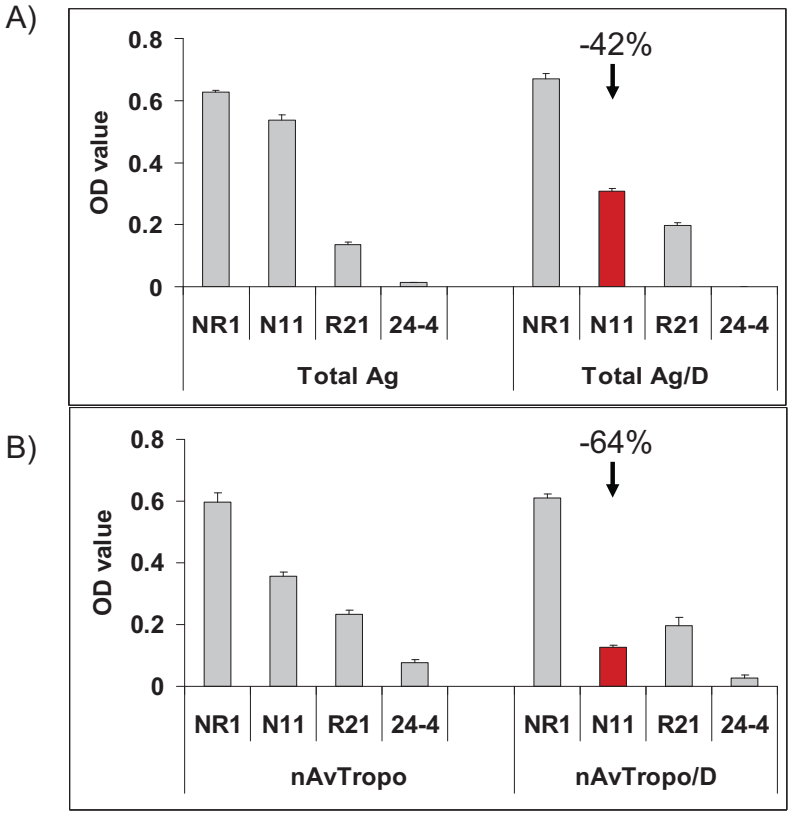


Figure 27. Influence of a metaperiodate treatment of antigens on binding of mAbs, as shown by ELISA. **A)** Female *Acanthocheilonema viteae* PBS extract (Total Ag) was used as a coating antigen before and after deglycosylation (Total Ag/D). **B)** affinity purified native tropomyosin (nAvTropo) was used as a coating antigen before and after deglycosylation (nAvTropo/D).

Comparison with untreated protein and worm extract revealed substantial loss of binding to the deglycosylated material (42 % in case of worm extract and 64% in case of a native protein). Experiment showed that deacetylation of both native protein and worm extract led to the lower binding of N11. Thus, results augment the hypothesis that N11 mAb is directed against an epitope formed entirely or in part of posttranslational modifications.

2.9.4. Tropomyosin is a surface component of A. viteae L3

A. viteae tropomyosin is an immunogenic antigen and interacts with the elements of the host immune system. However, it is unclear in where tropomyosin comes into contact

with host cells. To asses this, mAb NR1 was used in localization experiments with the L3 larvae. Results showed that NR1 antibody bound to the surface of L3 on the whole length of the parasite (Fig. 28 B). The intensity of binding was similar on the entire length of the L3 as no places with higher fluorescent signal levels were detected. Neither 24-4 antibody nor pooled serum of naïve BALB/c mice used for control bound to the surface of the parasite larvae (Fig. 28 D). Interestingly, no binding of any antibodies was detectable on blood microfilariae (not shown). The experiment showed that tropomyosin is located on the surface of infectious stage L3.

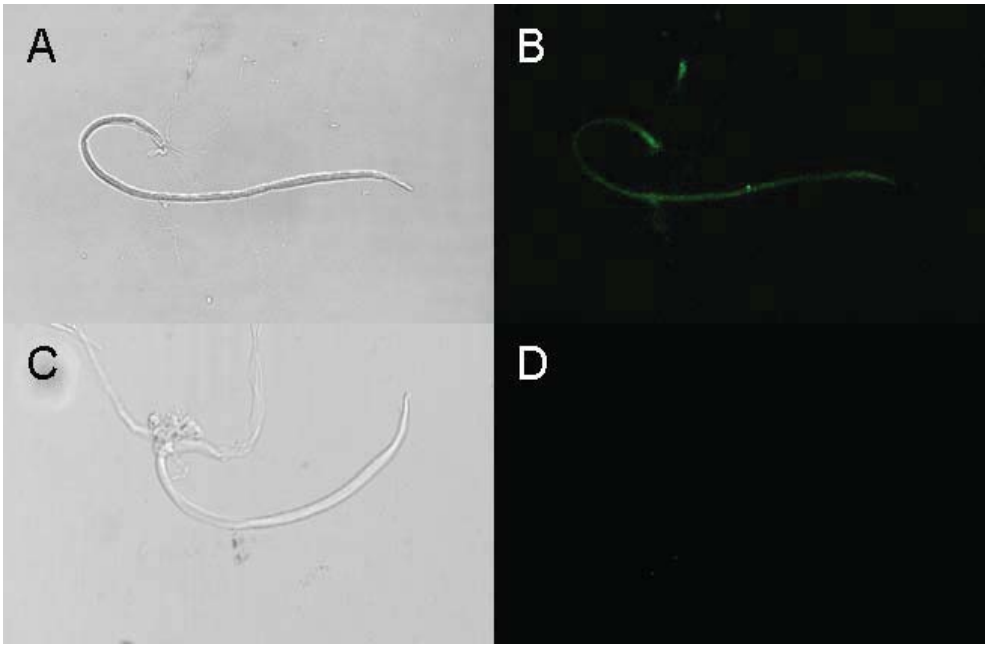


Figure 28. Localization of tropomyosin on the surface of *Acanthocheilonema viteae* L3 larvae shown by indirect fluorescence. NR1 (IgG1) mAb and FITC labeled secondary antibody were used in comparison to the isotype control mAb 24-4. **A)** NR1 labeled *A. viteae* in normal light. **B)** Green laser picture from confocal microscopy. **C)** Parasite L3 with 24-4 mAb in normal light **D)** picture C in green laser. Magnification 150 x.

2.9.5. Tropomyosin specific mAb help to kill microfilariae in vitro *by ADCC*

The functional ability of specific monoclonal antibody to bind *A. viteae* tropomyosin was also tested in assays utilizing antibody dependant cell cytotoxicity (ADCC). Microfilariae and L3 of *A. viteae* were incubated together with mouse adherent peritoneal exudates cells, mostly macrophages. Cells were incubated together with parasites while medium was supplemented with NR1, R21, N11 or 24-4 and 1E5 control mAb. Serum from

hamster challenged repeatedly with microfilariae or sera of jirds infected with L3 were used as positive controls for the experiment.

In all experiments, the antibodies did not mediate the adhesion of cells to the surface of L3 after 24 or 72 hours of culture. Parasites were alive and fully motile (not shown). Cell adhesion to mf and deterioration of parasites were observed in microfilariae experiments after 24 hours of incubation. In the positive control culture with hamster serum (Fig. 29 A and B) many larvae were trapped by cell clumps or covered with attacking cells. Bodies of larvae showed numerous vacuoles. Parasites were elongated and move rarely and slow (Fig. 29 B). Similar effects were observed when cells were supplemented with infected jird sera and NR1 mAb (Fig. 29 C and D, respectively). The processes of antibody dependant microfilariae killing induced by hamster serum or NR1 mAb led to the killing of last parasites within 72 hours of coculture (Fig. 29 E and F).

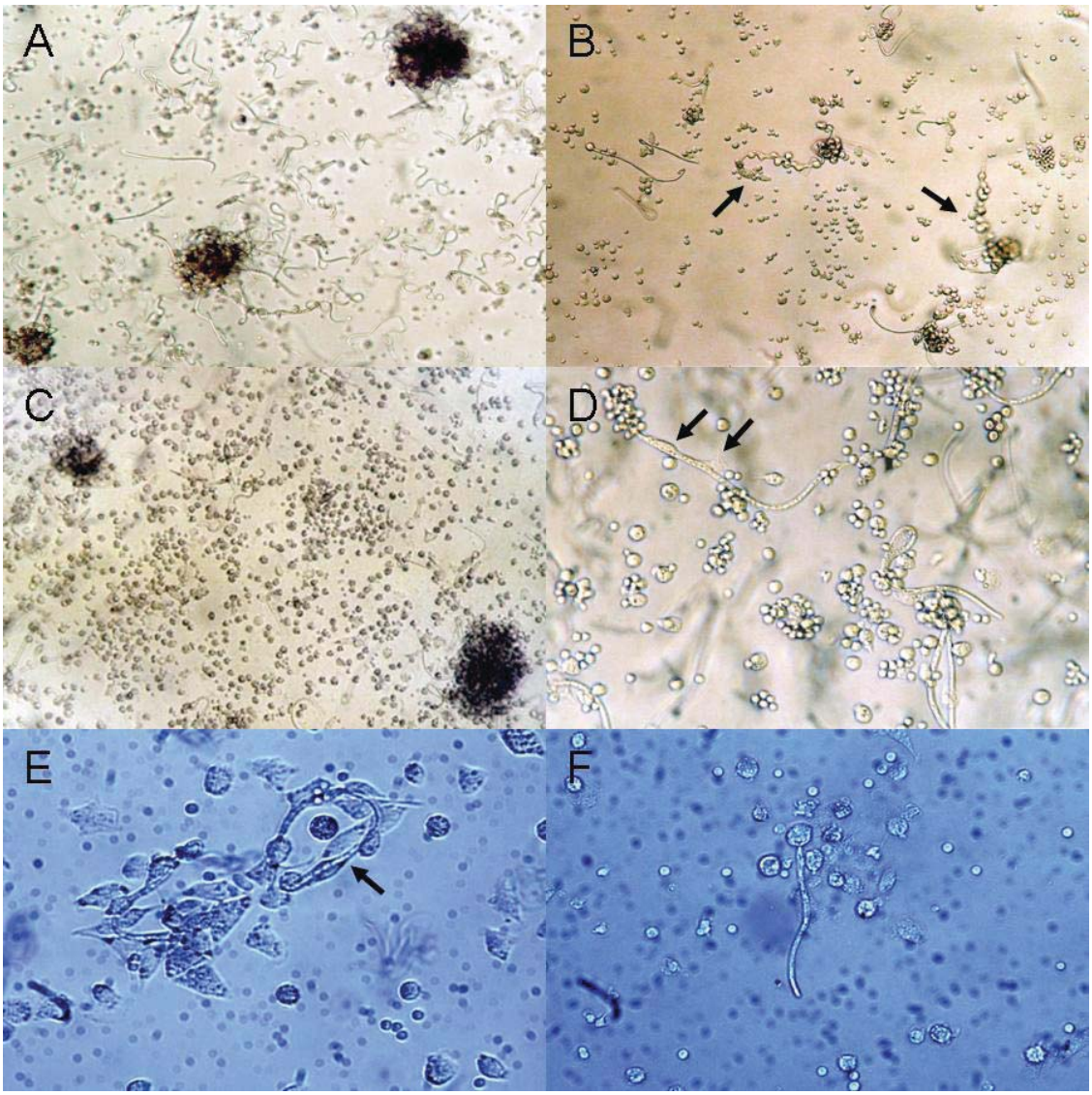


Figure 29. mAb against *A. viteae* tropomyosin participate in ADCC processes against microfilariae. **A)** Mouse macrophages supplemented with serum of hamsters immunized with mf. Clumps of cells around incapacitated mfs, after 24 h of incubation, 125x; **B)** Macrophages supplemented with immunized hamster serum cultured with mfs after 24 h of incubation. Arrows point attacked larvae, 125x; **C)** Macrophages and mfs cultured with an addition of serum of infected jirds, 24 h, 125x; **D)** Macrophages and mfs culture after 24 h incubation with NR1 mAb. Arrows point out cells attaching to the parasite, 250x; **E)** Macrophages and mf culture supplemented with hamster serum after 72 h, arrow indicates the position of mf, 400x; **F)** Macrophages and mfs together with NR1 mAb after 72 h incubation. One of the last microfilariae attacked and partially immobilized by macrophages, 250x.

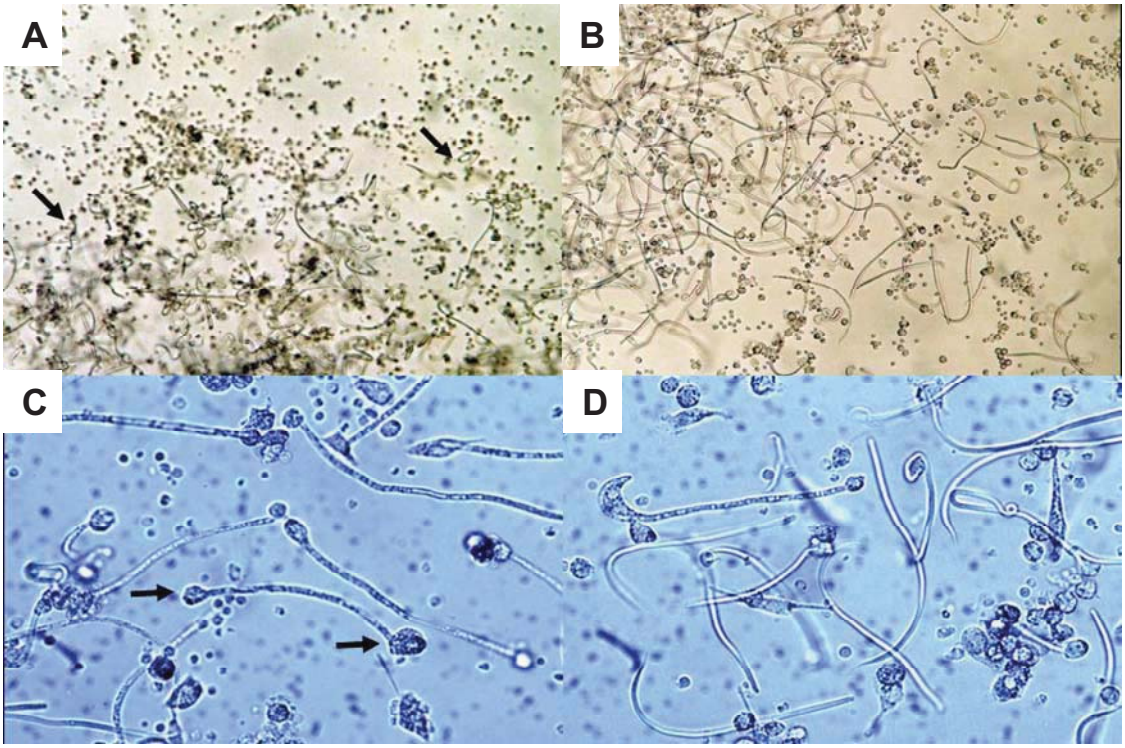


Figure 30. mAb against *Acanthocheilonema viteae* tropomyosin participate in ADCC processes againat microfilariae. **A)** Macrophages and mf coculture supplemented with R21 mAb 24 h, No effect on parasite larvae. **B)** Macrophages + 1E5 control mAb 24 h, No effect on parasite larvae. **C)** Macrophages Supplemented with 24-4 antibody 72 h **D)** Macrophages supplemented with N11 antibody. 24 h.

In contrast, all microfilariae were healthy and no cell adhesion to parasites or clumps were observed in approaches with R21 mAb and 24-4 control (Fig. 30 A and B). Interestingly, after 24 hours incubation no adhesion of cells to mf in culture supplemented with N11 antibody was detected. However, many parasites were less motile or did not move. After 72 hours macrophages supplemented with N11 antibody also showed ADCC like activity and attacked mfs (Fig. 30 C), many of them were full of vacuoles. At the same time in cultures supplemented with R21 and 24-4 control the majority of parasites were as healthy and agile as control parasites cultured with macrophages without addition of any antibody. Macrophages sporadically attacked mf in 24-4 experiment (Fig 30 D).

These results point out that microfilariae have tropomyosin on their surface and that the specific anti-tropomyosin mAb used in the experiment can bind to it. However, within the time of experiment (72 hours coculture) similar processes in regard to L3 had very

limited or no effect, showing that antibody dependent mechanisms may not play a crucial role in the killing of this larval stage of parasite.

2.10. T cell responses to tropomyosin

Tropomyosin of the shrimp *P. aztecus* not only induces B cell responses, but is also capable of inducing highly specific T cell responses. It was shown that some T cell epitopes can induce T cell proliferation of a level similar to the whole protein. Therefore, we tested both forms of *A. vitae* tropomyosin along with several synthetic peptides derived from its sequence. Moreover, such an induction augmented with the adjuvant can evoke a shift in the cytokine secretion, leading to the dampening of a Th2 phenotype.

2.10.1. Both, the whole molecule and synthetic peptides based on A. viteae tropomyosin are capable to stimulate T cell proliferation

To determine whether AvTropo stimulates T cell responses we used BALB/c mice that were immunized with eAvTropo or rAvTropo together with STP and restimulated with different antigens or single synthetic peptides bearing putative T cell epitopes. Results show that both eAvTropo and rAvTropo have T cell stimulatory capacity as spleen cells of mice immunized with both forms of antigen, upon restimulation with different tropomyosins, mounted a proliferative response that was 3 to 5 fold higher than the control (Fig. 31). The intensity of proliferation was independent of the form of tropomyosin used. Both rAvTropo and nAvTropo were able to restimulate splenocytes from mice immunized with a different form of tropomyosin. The T cell proliferation was specific for AvTropo as a recombinant control protein (rDHFR) and control synthetic peptide failed to activate spleen cells from TM immunized mice.

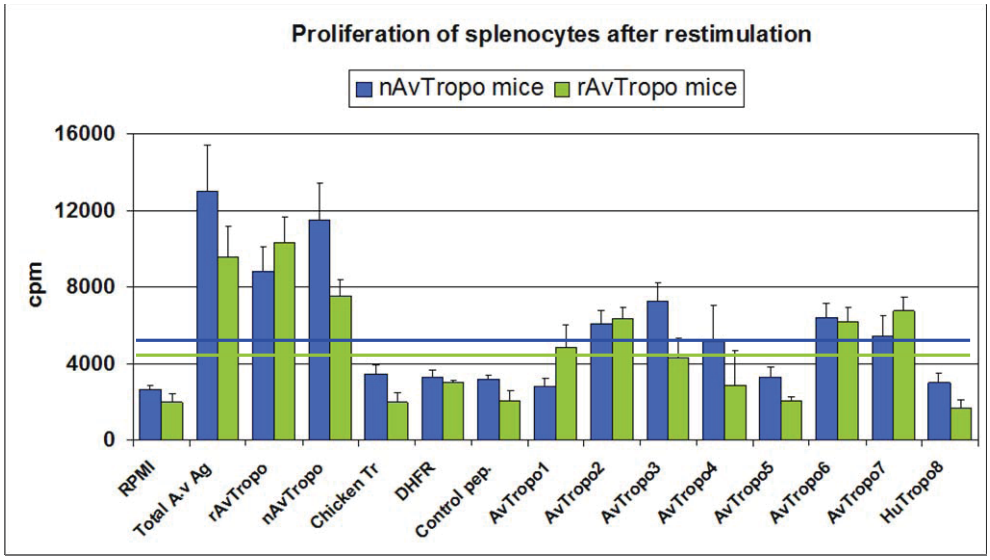


Figure 31. Proliferation tests of splenocytes from mice immunized with rAvTropo or nAvTropo and restimulated with different antigens or synthetic peptides. Blue and green lines represent threshold levels (2 x value of control + SD).

Synthetic peptides comprising putative T cell epitopes of *A. viteae* tropomyosin were also tested on splenocytes from immunized mice. Peptides A1 to A7 that were originally used for IgE epitopes analysis also comprise putative T cell epitopes (a result that corresponds to the sequence analysis with the Rammensee algorithms that detected 34 putative T cell epitopes on *A. viteae* tropomyosin with use of BALB/c haplotypes *H2-Ak* and *H2-Ek*).

Results of the experiment showed that the splenocytes could be restimulated with synthetic peptides although not as effective as with a whole molecule. Splenocytes from mice immunized with nAvTropo could be restimulated by peptides from middle or C-terminal part of tropomyosin molecule Av2, Av3, Av6, Av7 (threshold = 2 x the value of control). In comparison, cells from animals immunized with rAvTropo were stimulated to growth only by peptides A2, A6 and A7. These peptides were evoking increased T-cell proliferation and were more potent in inducing of cell proliferation than peptide A1 from N-terminus or peptides A4 and A5 from the middle of the molecule. The data suggest that peptides A2, A6 and A7 contain functional T cell epitopes. These epitopes are localized in the middle and C-terminal part of on *A. viteae* tropomyosin (Fig. 31).

2.11. A. viteae tropomyosin influences expansion of various cell populations after immunization

After experimental vaccination with *A. viteae* tropomyosin alone without any adjuvant we observed a high specific antibody response against this antigen. Sera of mice treated with filarial tropomyosin showed among others high levels of IgG1, IgG3 and IgE antibodies, a response strongly biased towards the Th2 phenotype. This situation is reminiscent to the effects of immunization with aluminium hydroxide (alum). To examine how tropomyosin can influence the production of antibodies and what cell populations take part in this process we started the series of experiments comparing *A. viteae* tropomyosin with alum adjuvant and a control protein.

2.11.1. Splenocytes from animals immunized with A. viteae tropomyosin produce IL-4 and IL-10 but no INFγ

Splenocytes from mice immunized with rAvTropo, affinity purified *A. viteae* tropomyosin (nAvTropo) and mice immunized with rDHFR 6 days after vaccination were tested for production of specific cytokines to asses their influence on production of cytokines. Cells were restimulated with antigens in a concentration of 5 µg / ml for 120 hours. Supernatants from cells were tested in capture ELISA test for the cytokines IL-4, IL-10 and INF-γ.

Restimulation of cells with nAvTropo lead to the release of both IL-4 and IL-10 on moderate levels (ca 400 pg / ml), while recombinant antigen induced no IL-4, but lead to a similar production of IL-10 (ca. 400 pg/ ml, Fig. 32 A and B). At this time no INF-γ was detected. Results were tropomyosin specific as cells from the same animals restimulated with rDHFR control protein did not produce cytokines or produced them on significantly lower level (data not shown). Moreover, cells of PBS treated animals also did not react to filarial tropomyosins and rDHFR.

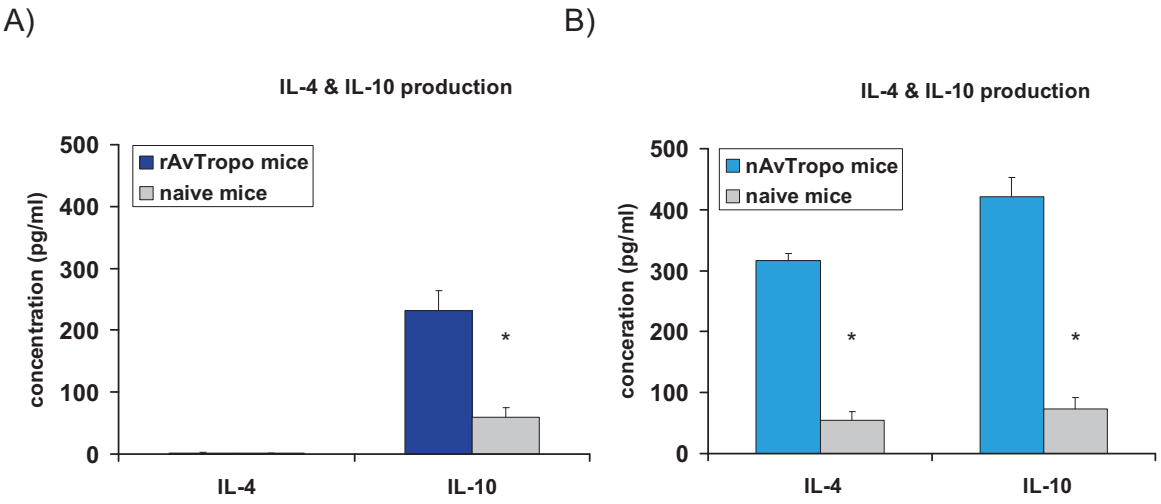


Figure 32. Production of IL-4 and IL-10 by splenocytes of naïve and rAvTropo or nAvTropo immunized BALB/c mice, as shown by cytokine ELISA. **A)** Cells stimulated with rAvTropo. **B)** Cells stimulated with nAvTropo. 3,5 x 10^5 cells per well were restimulated for 5 days. Average level of cytokine release in pg with SD is shown. Asterix indicates statistically significant difference ($p < 0.05$).

These results show that native *A. viteae* tropomyosin has an intrinsic capability to stimulate the production of both IL-4 and IL-10. The release of IL-4 is in line with the observation of IL-4 release in animals immunized with alum and could be directly connected with the high level of the antibody response. However, experiment also showed that both native and recombinant tropomyosin led to the production of IL-10, a cytokine associated with the suppression of cell activity and down-regulation of some immune system elements. In the cause of rAvTropo the production of IL-4 after 5 days of the culture was not observed.

2.11.2. Immunization with A. viteae tropomyosin expands Gr1$^+$/CD11b$^+$ cells in spleen

The intraperitoneal application of alum was shown to induce the expansion of the population of Gr1$^+$/CD11b$^+$ cells in the peritoneal cavity and spleen of treated animals where they facilitated IL-4 dependent B cell priming and production of specific antibody (Jordan et al., 2004). The surface molecule Gr1 is a marker expressed on immature myeloid cells as well as mature granulocytes and was mainly discovered on the surface of neutrophils, eosinophils and basophils, while CD11b is a marker on the surface of myeloid cells including macrophages and monocytes. The following experiment

addressed the question if a similar upregulation of $Gr1^+/CD11b^+$ subpopulation takes places after immunization with filarial tropomyosin. It turned out that application of *A. viteae* tropomyosin resulted in a significant increase of $Gr1^+/CD11b^+$ in spleens analysed 6 days after treatment (Fig. 33). Application of rAvTropo led to increase of this subpopulation between 12 and 25%, while nAvTropo induced levels between 12-20%. Results obtained for filarial tropomyosin were very similar to those observed for alum (12-24, Fig. 33).

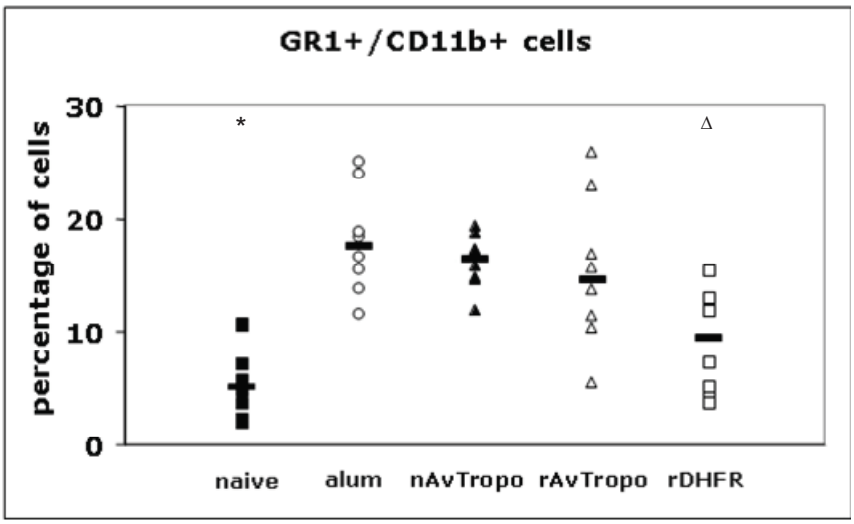


Figure 33. Level of $Gr1^+/CD11b^+$ cells in spleens of immunized C57/BL6 mice. Groups of mice were immunized once with 100 µg of pure protein in PBS, or 250 µl of PBS or alum intraperitoneally. Cells were harvested from spleens 6 days after immunization. Graph shows the percentage of $Gr1^+/CD11b^+$ in splenocytes from individual mice after immunization with various antigens. Black bars show median for each group. Data shown are derived from two independent experiments. Asterix and delta represent statistically significant difference ($p < 0.05$).

In comparison, treatment of animals with PBS or a control protein rDHFR did not lead to a significant increase of $Gr1^+/CD11b^+$ cells (naïve animals: 2-11%, rDHFR: 4-15%). The cell numbers for tropomyosin and alum groups were significantly higher that those obtained from naïve and rDHFR control animals ($p < 0.05$). Thereby, treatment with filarial tropomyosin resulted in a comparable increase of this cell population as seen after alum treatment.

Additional results showed that both native and recombinant filarial tropomyosins used in this study are potent inducers of a $Gr1^+/CD11b^+$ subpopulation of cells in spleens of

immunized animals. It corroborates the idea that tropomyosin has substantial influence on the host immune system, inducing influx and propagation of $Gr1^+/CD11b^+$ cells (Fig. 34). The expansion of the nominated cell subpopulation also suggests that the induction of antibody response after tropomyosin immunization corresponds to the similar patterns observed for alum.

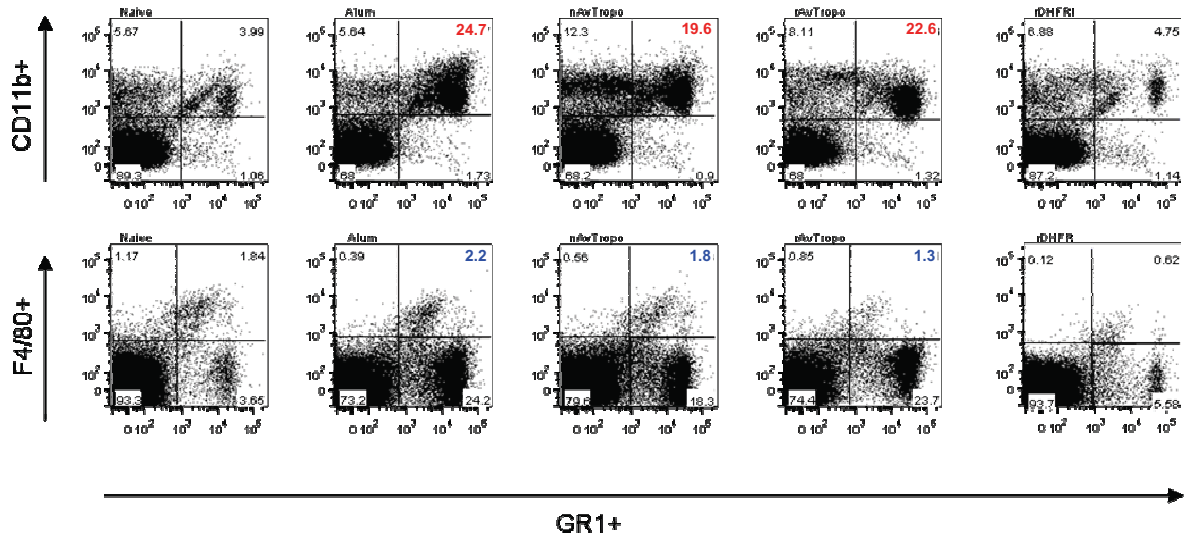


Figure 34. Flow cytometry of spleen cells of immunized C57/BL6 mice. Groups of mice were immunized once with PBS (naïve), 250 µl alum or with 100 µg of pure protein in PBS intraperitonealy. Cells were harvested from spleens 6 days after immunization and stained for $Gr1^+$, $CD11b^+$, $F4/80^+$. Representative dot plots are shown.

Data from animals immunized with *A. viteae* tropomyosin, but not these treated with alum or rDFHR or naive indicated higher proliferation of $CD11b^+$ cells (Fig. 34 upper panel). Moreover, by staining cells with antibody against macrophage specific surface marker F4/80 we determined the number of macrophages within this subpopulation. However, as shown in Fig. 34 (lower panel), within the population of $Gr1^+/CD11b^+$ in spleen there were no significant changes of macrophages ($F4/80^+$) populations between naïve and immunized animals. The majority of $Gr1^+$ expressing cells did not co-express F4/80 and should thereby be regarded as either granulocytes or immature myeloid cells.

2.11.3. Gr1$^+$/CD11b$^+$ subpopulation of splenocytes is responsible for production of IL-4 and IL-10

It was already shown that the splenocytes from mice immunized with rAvTropo or nAvTropo produce IL-4 and IL-10 after restimulation *in vitro*. To investigate whether Gr1$^+$/CD11b$^+$ subpopulation contributes to this production cells expressing these markers were isolated from splenocytes of immunized animals and restimulated. Various antigens were used for immunization of mice: nAvTropo, rAvTropo, rDHFR and nAv83 (an electro-eluted control protein for nAvTropo). For restimulation antigens were added to the medium in concentration of 5 µg / ml and cell culture supernatant was collected after 72 hours.

Similarly as in the study with whole splenocytes (see Fig. 32) both nAvTropo and rAvTropo led to a release of IL-10 by the Gr1$^+$/CD11b$^+$ cells (Fig. 35 A and B). This time however, it could be attributed to the upregulated Gr1$^+$/CD11b$^+$ subpopulation found in splenocytes of immunized animals. Restimulation showed that both forms of tropomyosin led to a significantly higher production of IL-10 as compared to the control proteins.

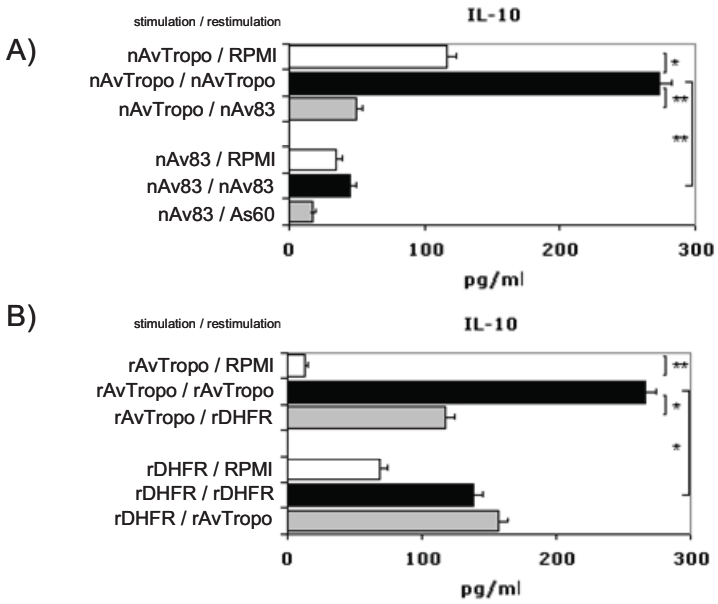


Figure 35. Production of IL-10 by Gr1$^+$/CD11b$^+$ cells from immunized C57/BL6 mice restimulated with various antigens. Groups of mice were immunized once with 100 µg of pure protein in 250 µl of PBS intraperitoneally. Cells were harvested from spleens 6 days after immunization. Bars show the average cytokine release of three animals. **A)** Comparison of IL-10 production between Gr1$^+$/CD11b$^+$ cells from spleens of nAvTropo and nAv83 immunized animals. **B)** Comparison of IL-10 production between Gr1$^+$/CD11b$^+$ cells from spleens of rAvTropo and rDHFR treated mice.

nAvTropo was able to induce the production of roughly 300 pg / ml IL-10, which was significantly higher than the effect of native control proteins (nAv83 and As60) and RPMI ($p < 0.01$). Similarly, $Gr1^+/CD11b^+$ cells from animals immunized with rAvTropo produced significantly more IL-10 than splenocytes restimulated with control proteins ($p < 0.05$). These results clearly showed that the $GR1^+/CD11b^+$ cells induced by the nAvTropo or rAvTropo immunization produce IL-10, thus tropomyosin has an intrinsic capability to induce IL-10 production.

The production of IL-10 by $Gr1^+/CD11b^+$ derived from animals treated with nAvTropo was accompanied by release of IL-4 after restimulation (Fig. 36 A). nAvTropo stimulates $Gr1^+/CD11b^+$ cells to produce significantly higher levels of IL-4 that all control proteins (nAv83, nAs60 and rDHFR, $p < 0.01$). However, rAvTropo did not induce IL-4 production by these cells (Fig. 36 B). These data suggest that the native form of *A. viteae* tropomyosin has some qualities lacking on the recombinant form. The discrepancy in the ability to stimulate IL-4 responses between these two forms can probably be attributed to the presence of the posttranslational modifications on the affinity purified nAvTropo.

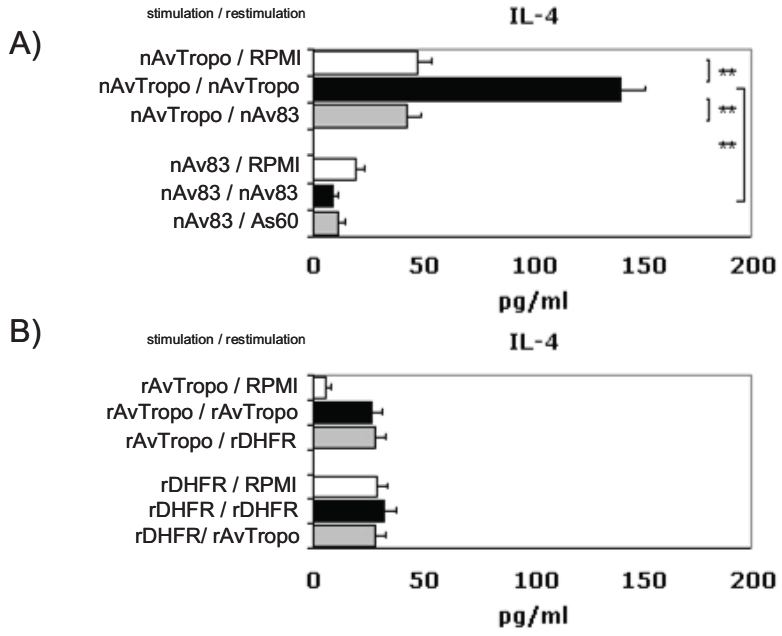


Figure 36. Production of IL-4 by $Gr1^+/CD11b^+$ cells from immunized C57/BL6 mice restimulated with various antigens. Different groups of mice were immunized once with 100 µg of pure protein or sham treated with 250 µl of PBS intraperitoneally. Cells were harvested from spleens 6 days after immunization. Bars show an average cytokine release of three animals. **A)** Comparison of IL-4 production between $Gr1^+/CD11^+$ cells from spleens of nAvTropo and nAv83 immunized animals. **B)** Comparison of IL-4 production between $Gr1^+/CD11^+$ cells from spleens of rAvTropo and rDHFR treated mice.

However, it can not be excluded that other circumstances play a role, for instance in vaccination experiments antigens were delivered subcutaneously and it is possible that the different route of antigen delivery resulted in presentation of tropomyosin to other cell populations that were responsible for IL-4 production initializing antibody production.

2.11.4. The subpopulation of $Gr1^+/CD11b^+$ cells rises in peritoneal exudate after immunization with rAvTropo

Previous experiments showed that numbers of cells expressing $Gr1^+$ and $CD11b^+$ cell markers greatly increased in spleen 6 days after animals were injected with *A. viteae* tropomyosin. These changes probably reflect an increase of granulocytes and/or immature myeloid cells as we did not detect changes of percentages of $F4/80^+$ expressing mature macrophages. Since mice were immunized intraperitoneally in all experiments we also investigated changes in the cellular composition of the peritoneal exudates (PE) shortly after the injection. To address this issue the PE cells were examined 18 hours after the immunization. Results showed that the subpopulation of $Gr1^+/CD11b^+$ cells appeared also in the peritoneum of animals injected with rAvTropo. Cells expressing $Gr1^+/CD11b^+$ comprised up to 25% of PE cells in animals injected with rAvTropo, while in PBS treated animals or animals which were given the control protein rDHFR this proportion was 4.3% and 4.8%, respectively (Fig. 37). The number of F4/80 cells expressing $Gr1^{+high}$ increased, while the percentage of cells expressing medium F4/80 dropped. In addition, the $CD11b^+$ cells present in naïve animals in 2 major subpopulations divided into 4 in respect of the level of $Gr1^+$ expression (high, $medium^+$, medium and low, Fig. 37).

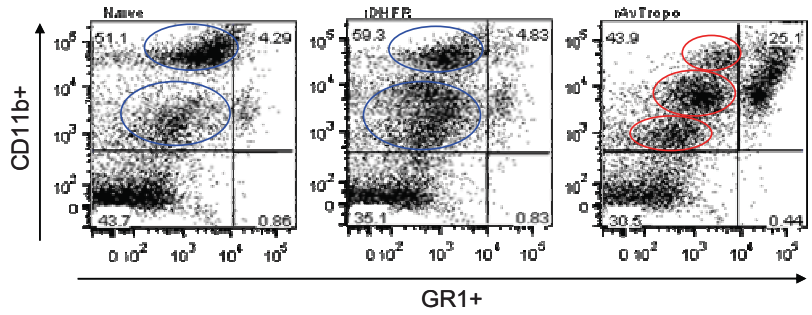


Figure 37. Level of $Gr1^+/CD11b^+$ cells in PEC of immunized C57/BL6 mice. Different groups of mice were immunized once with 50 µg of pure protein or 250 µl of PBS intraperitoneally. Cells were harvested from peritoneal cavity 18 hours after immunization. Dot plots show ex vivo populations in FACS analysis in respect to the surface markers. Distinct subpopulations of $Gr1^+/CD11^+$ cells were circled.

The short time span between application and detected changes in cellular composition argues for either the upregulation of $Gr1^+/CD11b^+$ markers on resident cells or the recruitment of new cells into the peritoneal cavity. The latter explanation is corroborated by the data obtained by cytospins of PE cells showing marked increase of neutrophiles and the higher absolute cell numbers found in peritoneum after the treatment (see Fig. 39).

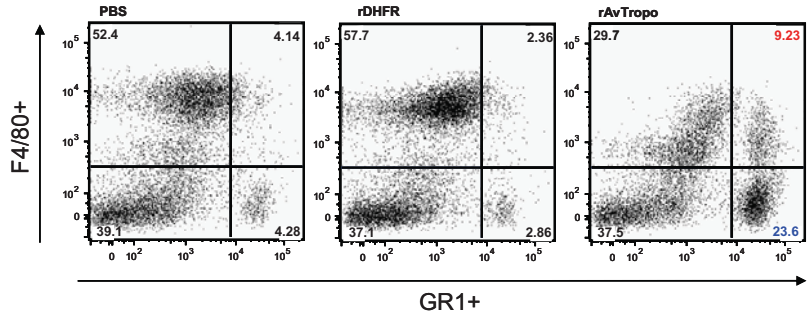


Figure 38. Level of $Gr1^+/F4/80^+$ cells in PEC of immunized C57/BL6 mice. Different groups of mice were immunized once with 50 µg of pure protein or 250 µl of PBS intraperitoneally. Cells were harvested from peritoneal cavity 18 hours after immunization. Dot plots show ex vivo populations in FACS analysis in respect to the surface markers.

On the other hand, expression of F4/80 marker within the total PEC population was observed to decline in rAvTropo treated animals. The proportion of $F4/80^+$ cells constituted 52.4% (PBS), 57.7% (DHFR) and 29.7% (rAvTropo) of the total cell population. Moreover, the subpopulation recruited into the peritoneal cavity 18 h after immunization contained a greater number of $Gr1^{+high}$ cells expressing the macrophage

cell surface marker F4/80. 9.2% of PEC from animals given rAvTropo were F4/80^{+high}, while only 4.1% and 2.3% of F4/80^{+high} cells appeared in PBS and control protein animals, respectively (Fig. 38). Thus, we observed that the immunization with filarial tropomyosin changes dramatically the constitution of PE cell populations. As compared with naïve animals it induced an influx of Gr1^{+high}/CD11b^{+} cells in great numbers (up to 25%) and changes in macrophage subpopulation. These changes upon contact with tropomyosin were possibly one of the first steps on the pathway resulting in the appearance of a Gr1^{+}/CD11b^{+} population of IL-10 producing cells in the spleen.

2.11.5. The constitution of PECs after immunization with A. viteae tropomyosin

To substantiate the data obtained by FACS analysis, PE cell populations of the animals treated with rAvTropo were characterized by cytospin.

The PE cell composition of rAvTropo injected animals was compared to PBS and rDHFR controls, and in addition to animals injected with LPS in a dose corresponding to the content of LPS in rAvTropo. Analysis revealed differences in cell populations 18 hours after the injection (Fig. 39). Results showed an increase in numbers of neutrophils (12-39%, median: 32%, $p < 0.01$ in comparison to PBS), eosinophils (1-9%, median: 4%) and basophils/mast cells (1-5%, median: 3%) in rAvTropo treated animals. Interestingly, a decrease in number of lymphocytes (8-27%, median: 14%) and macrophages (37-51%, median: 47%) was observed in comparison to control animals. Moreover, the number of neutrophiles differed between animals treated with rAvTropo and the LPS ($p < 0.01$), showing that the effect of filarial tropomyosin was not due to LPS contamination. These data support findings from the flow cytometric analysis, where higher frequencies of cells expressing GR1 marker on their surface and a decrease in F4/80^{+} macrophages were observed.

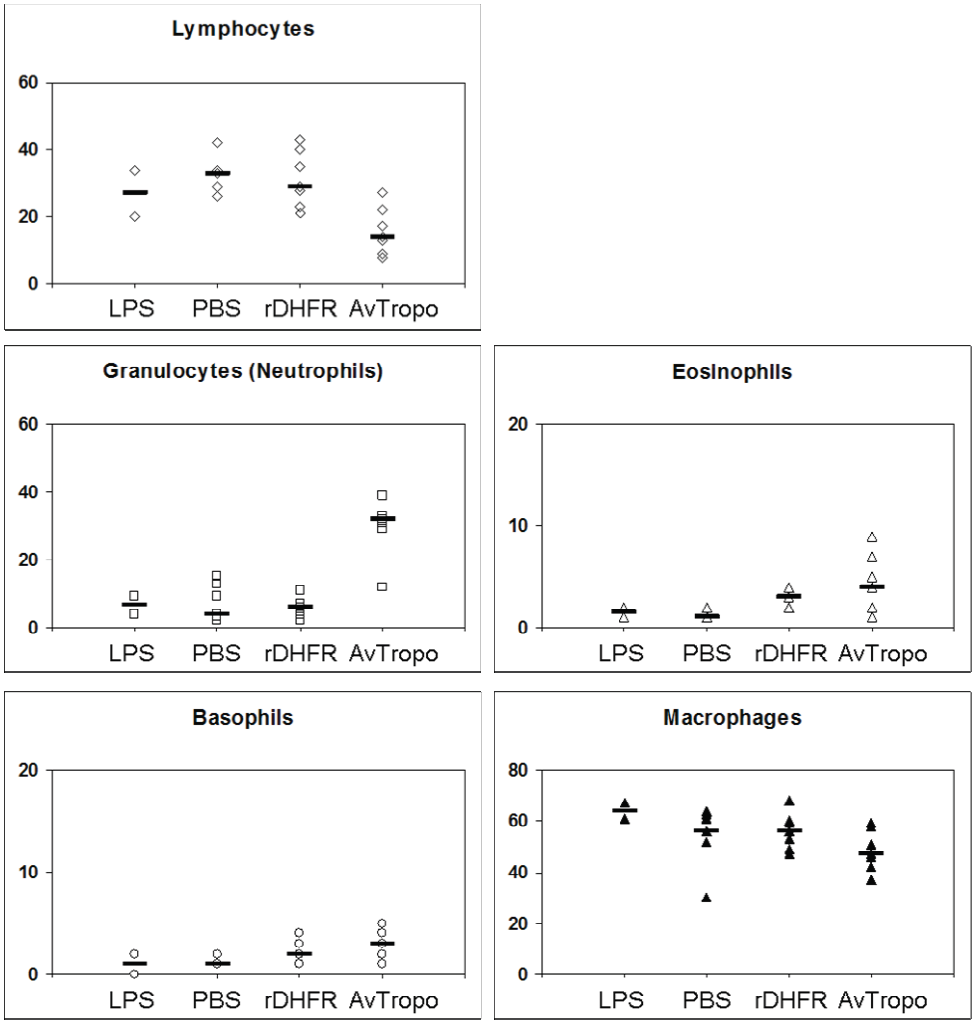


Figure 39. Changes in the constitution of PEC population after injection of rAvTropo in C57/BL6 mice as determined by the cytospin. Groups of mice were immunized once with 25 µg of pure protein, 250 µl of PBS or 2.5 ng of LPS in PBS intraperitoneally. Cells were harvested from the peritoneal cavity 18 hours after immunization. Black bars represent medians. Pooled results from two experiments with 4 and 2 animals.

3. Discussion

3.1. Tropomyosin of A. viteae as a vaccine

Numerous studies presented the view that establishing of protective immunity against the filarial nematode *O. volvulus* is possible. Such opinion is based on studies on humans in endemic areas that provided evidence that between 1% and 5% of population acquired a natural immunity (Ward et al., 1988; Elson et al., 1994). These naturally protected individuals were named putatively immune (PI) (King, 2000; Ravindran, 2001). These patients do not develop filarial infection and they do not suffer from the disease. It was also found that sera from PI induce *in vitro* opsonisation and killing of L3 and negatively influence moulting of L3 to L4 (Greene et al., 1985; Johnson et al., 1994). Based on the cited observations it is clear that understanding of the immunological mechanisms taking place in PI individuals and elaborating them in animal models could help in development of successful vaccines.

There are conflicting views on the key mechanisms directing development of protection against various developmental stages of the parasite. Some studies suggested that protective immunity in PI individuals is dependent on diminished specific IgE and IgG responses and an enhanced production of IL-2 and INF-γ in response to antigens of adult worms. This implies that protective immunity may be related to the Th1 dependent arm of the immune response (Lüder et al., 1996; Soboslay et al., 1997; King, 2000). However, in other studies concerning PI, protective immunity was suggested to be due to mixed Th1 and Th2 or dominant Th2 responses and production of IL-2, IL-5 and GM-CSF (Brattig et al., 1997, Turaga et al., 2000). Similarly, research in mouse models of filariasis led to the observation that antibodies associated with Th2 response, directed towards the surface of L3 and the microfilarial sheath (Eisenbeiss et al., 1994) play a role in limiting of the larval development. Moreover, use of L3 implanted in diffusion chambers (Lange at al., 1993) demonstrated a Th2 profile of immunity and showed dependence of partial protection on IL-4, IL-5 and IgE. These findings were corroborated by cytokine depletion experiments and vaccination of IL-4 and INF-γ knockout mice (Lange et al., 1994; Johnson et al., 1998). Additionally, studies on jirds vaccinated with irradiated larvae of *A. viteae* (Bleiss et al., 2002) and in a mouse model (Abraham et al.

2004) showed that protection against L3 can be attributed to the presence and degranulation of eosinophils in the vicinity of L3. Latter study showed also that mice lacking EPO (eosinophil peroxidise) or IgE were unable to develop protective immunity to parasites.

Experimental studies showed that L3 could be promising sources of different antigens for the development of effective vaccines (Lucius et al., 1991; Lok and Abraham, 1992; Eisenbeiss et al., 1994). Partial protection against challenge was achieved by immunization with irradiation-attenuated L3 in animal models like jirds, mice and chimpanzees (Lucius et al., 1991; Lange et al., 1993; Prince et al., 1992; Le Goff et al., 1997). Also immunization with normal larvae conferred protection (Denham et al., 1983; Lucius et al., 1986; Eisenbeiss et al., 1994). However, the extent of this protection varied between 30% and 90% depending on the host parasite system studied and particular conditions. More recently, recombinant *O. volvulus* larval antigens were also used for vaccination (Jenkins et al., 1996; Taylor et al., 1996) showing a moderate level of protection.

Tropomyosin could be considered as an attractive anti-filarial vaccine candidate. There are two reasons that predestine this protein to be a good antigen. Firstly, it is present in important developmental stages, relevant in respect of vaccine efficacy and infection prevention. As shown in *O. volvulus* filariae it is found in both infective L3 and microfilariae and is expressed not only in muscles but also on the surface of the cuticule (Jenkins et al., 2000). Apart of filariae, tropomyosin was also located on the surface of L3 of the intestinal nematode *Trichinella spiralis* (Nakada et al., 2003). Thus, tropomyosin represents a target for the immune responses directed against parasitic nematodes. Secondly, it was observed that *A. viteae* tropomyosin was a target for monoclonal antibodies derived from animals challenged repeatedly by implantation of irradiated L3 (Hartmann et al., 1997), showing that this protein can induce potent antibody responses. It was also found by immunoblot in all developmental stages of *A. viteae* including L3 and mf.

Vaccination experiments described in this work showed that triple immunization with recombinant filarial tropomyosin imparts partial protection in jirds against challenge with

L3 of *A. viteae* but only when applied within a protocol that favors the development of Th1 responses. Immunization with recombinant or natural, worm-derived tropomyosin induced about 30% protection when STP was used as an adjuvant - a mixture of chemicals that primarily stimulates Th1 responses (Cox et al., 1997). Results are in good line with the data published so far.

Immunization with native tropomyosin was effective against *T. colubriformis* L3 challenge in a guinea pig model, reducing worm burdens by 43-51% (O'Donnell et al., 1989). Immunization of BALB/c mice with a C-terminal fragment of *O. volvulus* tropomyosin (MOv14) comprising 136 aa and expressed as a maltose binding protein (MBP) fusion evoked 48-62% reduction in the number of *O. lienalis* microfilariae. In this model, the challenge infection consisted in microfilariae of a cattle parasite intravenously inoculated into mice (Taylor et al., 1996). Interestingly, tropomyosin-associated protection against such microfilariae could be transferred with serum to naïve recipient BALB/c mice (Folkard et al., 1996). Similarly, jirds were protected by immunization with MOv14 against a challenge with L3 of the rodent filaria *A. viteae*, as adult worm burdens were reduced by 46% and the level of circulating microfilariae was reduced by up to 89% (Taylor et al., 1996). Tropomyosin of *A. viteae* had similar protective capacities as immunization of jirds with native tropomyosin induced up to 64% reduction in worm burdens with a corresponding 93% reduction in circulating microfilariae (Hartmann et al., 1997). Existing differences between results presented in this work and cited articles can be attributed to the different animals models, various vaccination schemes utilized and other read-outs for the experiments.

Similarly, intramuscular injection of AvTropo DNA – a route of immunization that elicits a Th1-polarized immune response (Morel et al., 2004) induced moderate protection against challenge infection. The efficacy of DNA vaccine used in this study was doubled (20% to 40%, $p < 0.05$) by the inclusion of aluminium phosphate as an adjuvant or by the introduction of prime-boost regime of vaccination, where DNA vaccine was followed by a boost with recombinant protein (45%, $p < 0.02$).

There are several possible reasons behind the significant increase in protection in DNA vaccine assays in comparison to protein based vaccines. While protein immunisation is

restricted to the Th2 directed responses, the intramuscular immunization with cDNA has been described to induce clear Th1 responses (Morel et al., 2004) partially due to unmethylated CpG motifs within bacteria-derived plasmids (Chu et al., 1997; Gurunathan et al., 2000). CpG directly stimulate B cells and plasmacytoid dendritic cells via TLR9, thereby initiating and promoting the development of Th1 cells, production of proinflammatory cytokines and the maturation/activation of professional APCs. This properties enable CpG to act as adjuvants and to accelerate and boost antigen-specific immune responses 5 to 500 fold (reviewed in Klinman et al., 2004). Interestingly, treatment of parasitic infections with CpG containing oligodeoxynucleotides showed that injection before infection with *Leishmania major* or *Plasmodium yoelii* could convert mice to a Th1-dominant resistant phenotype shifting production from IL-4 to INF-γ and IL-12 (Zimmermann et al., 1998; Gramzinski et al., 2001). Injection with CpG oligonucleotides was also described to limit Th2-mediated *Schistosoma mansoni* egg-induced pathology by upregulation of IL-10 (Chiaramonte et al., 2000). Additionally, aluminium phosphate adjuvant used in my trials (AdjuPhos) due to the positive electrical charge does not bind DNA (Kwissa et al., 2003) enabling perhaps a better recognition or uptake of DNA by cells at the site of injection. Moreover, other results indicated that aluminium phosphate increased the potency of DNA vaccines by adjuvant effects on the immune response to the antigen already expressed *in vivo* (Ulmer et al., 1999). The approach with prime-boost strategy (heterologous boosting) which utilized DNA vaccination followed by injection of a recombinant antigen was shown to generate high levels of memory T cells (reviewed in Woodland, 2004).

Previous studies with DNA immunisation were to some extent efficient against a variety of pathogens (Robinson and Torres, 1997). DNA vaccines have been shown to induce protective immunity against the intracellular protozoan parasites *Plasmodium falciparum* (Sedegah et al., 1994; Hoffman et al., 1997) and *Leishmania major* (Xu and Liew, 1994), where protection is mediated by Th1-like responses in conjunction with INF-γ upregulation. Interestingly, a partial protection against *O. volvulus* infection in mice was obtained when immunised with DNA coding for chitinase (Harrison et al., 1999) or tropomyosin (Harrison and Bianco 2000). In my study DNA vaccination was up to 9 fold more efficient than the standard *E. coli*-protein based approach.

All described schemes induced low or moderate quantities of serum IgM, IgG and IgE. In contrast, no reduction of worm burdens was seen when animals were immunized with tropomyosin emulsified in alum (5%), a treatment inducing Th2 responses with high levels of antibodies, in the mouse particularly IgG1 and IgE (Mancino and Ovary, 1980). The differences of IgG1 and IgE and IgG subclasses after immunization with alum in comparison with STP and DNA supports the idea of skewing of T helper cell responses by adjuvants or DNA. It is noteworthy that the proportion of IgG1 response (associated with Th2) to IgG2a (Th1) was measured to be 3.3:1.0 in rAvTopo/alum immunized animals, whereas in rAvTropo/STP and pcDNA/AvTropo jirds these relations were 2:1 and 1:4, respectively. Thus, my data suggest that immunization with STP induced a mixed type of response while DNA vaccination resulted in Th1 response.

Immunisation studies that compare *E. coli*-expressed proteins and native proteins have shown that native/natural antigens have higher capacity to raise protection (Newton and Meeusen, 2003). Recombinant proteins might be unsuitable because they could lack critical epitopes owing to improper folding of proteins or absence of post-translational modifications. However, in this study recombinant protein had the same effect on protection as natural one if both were applied under the same regime. Both approaches with STP resulted in around 30% protection. This might be due to the fact that the recombinant *A. viteae* tropomyosin has a proper folding and a natural/proper secondary structure of the molecule, as demonstrated by CD experiment.

Nonetheless, glycan epitopes as demonstrated by Vercauteren et al., (2004) may be targets of protective antibody responses against intestinal nematodes. Also studies with native H11 protein from *Haemonchus contortus* have determined that the 90% protection obtained is due to glycosylation (Newton and Munn, 1999). A similar situation was also observed in the case of *B. malayi* immunisation studies (Peralta et al., 1999) where native basement membrane collagen epitope (AP2) induced significant reduction in adult worm burden in comparison with *E. coli*-expressed protein**.** Additionally, it was described that soluble extracts of *B. malayi* and *C. elegans* induced antigen specific IL-4 production that was dampened when extracts were treated with sodium periodate – a treatment eliminating glycans (Tawill et al., 2004). Studies on egg-derived

glycoconjugates of *S. mansoni* showed that complex-type N-glycans induced a glycan specific Th2 cellular response with high levels of specific IgG1. These glycans contained the core α3-fucose and core β2-xylose determinants that were found on many others invertebrates (Faveeuw et al., 2003). Schistosome glycans were described to be associated with development of specific anti-carbohydrate IgE that are important in limiting reinfection in humans (van der Kleij et al., 1999; Nyame et al., 2000). Thus, post-translational modifications of helmith antigens could influence vaccination efficacy. In case of *A. viteae* tropomyosin computer prediction showed two putative sites, N^{132} and N^{269}, where N-glycan moieties can be localized. However, this information was not proved experimentally in this study and no literature exist on posttranslational modifications of tropomyosin in other species. Interestingly, it is believed that such epitopes might play an important role in allergenic properties of proteins.

Cited reports also employed immunization schemes that drove immune responses against tropomyosin into Th1 direction, either by using Freund's Complete Adjuvant, STP or intramuscular DNA vaccination (see Tab. 2). Thus, the published data and my observations are in line and support the link between Th1 immune responses and the protective immunity against L3. The interesting aspect of these observations is the fact that filarial infections are described to induce a polarization towards Th2 responses (Kurniawan et al., 1993; Bancroft et al., 1993; Tawill et al., 2004). Thus, tropomyosin is protective under conditions that do not correspond to the situation caused by the development of the natural infection. In contrast, an immunization scheme that drives responses into Th2 direction, immunization together with alum, was not protective at all.

This situation is reminiscent to immunizations with so called "novel antigens" or "concealed antigens", i.e. antigens that do not induce immune responses under conditions of a natural infection. Such antigens are typically localized in the gut of blood feeding helminths or ticks, structures to which the host immune system does not have any access. Concealed antigens have successfully been used as experimental vaccines against gastrointestinal nematodes like *H. contortus* (Newton and Munn, 1999). Interestingly, a commercial vaccine against the tick *Boophilus microplus* also relies on vaccination with a protein of the gut epithelium (Riding et al., 1994). It is possible that the efficiency of vaccines based on concealed antigens is owing to the fact that worms did

not develop evasion mechanisms during their co-evolution with the host that would counteract the immune effector mechanisms directed against these antigens. By analogy, an antigen that displays protective properties when applied in a context that does not occur during an infection, can also be considered as “novel antigen”. Therefore, this study shows a potentially important principle, and “novel antigens” of the tropomyosin type might be applied for the development of anti-helminth vaccines.

Observations suggest that protection of jirds against L3 does not depend on effector mechanisms involving antibodies. Low levels of antibody responses in jirds vaccinated with pcDNA/AvTropo as well as the lack of correlation between anti-tropomyosin IgG and IgE responses of rAvTropo-immunized jirds and protection on the group level and on the individual level suggest that antibodies were not involved in protection against challenge infection. This is in contrast to the general view that considers ADCC mechanisms as an important immune effector mechanism against parasitic nematodes (Lawrence, 2001). For example, the study by Abraham et al. (2004) shows that IgE and eosinophils are crucial components for the protective immune responses against L3 of *O. volvulus* in mice vaccinated with irradiated L3. Along the same lines, a recent publication of Paciorkowski et al. (2003) describes that primed B cells are sufficient for the development of immunity against challenge infections of mice with L3 of the filarial parasite *B. pahangi*. Moreover, it was shown that IgG1 deficiency increases susceptibility of mice for infection with L3 of *B. malayi* and that clearance of infection was correlated with an increase in the IgG1 titers in mice (Spencer et al., 2003). These statements do not directly contradict the fact that immunity induced by filarial tropomyosin would operate independent of antibodies. Immunity against large metazoan parasites like filarial L3 probably requires cooperation of different immune effector mechanisms, directed against various antigens, at different time points and sites to limit infections (Maizels and Yazdanbakhsh, 2003). Thus, immune effector mechanisms like the antibody-independent response shown here might co-exist with ADCC that is directed or more efficient against other developmental stage of the parasite (eg. mf instead of L3). Moreover, the previously cited articles analyze the reaction of mice against filarial parasites that are not fully adapted to this host species. Therefore, it is not excluded that

studies with such non-natural host-parasite associations are biased with regard to immune effector mechanisms (Meeusen and Balic, 2000).

This study showed that tropomyosin is not only present in PBS soluble extracts of *A. viteae* worms but also expressed on the surface of L3 and Mf as shown by the experiments with indirect immunofluorescence and induction of ADCC. Moreover, *A. viteae* tropomyosin specific mAb promote ADCC-associated killing and clearance of mf by macrophages *in vitro*. However, such results were not observed for L3 which under the same experimental constrains survived in a very good condition. On the other hand, mAb were able to bind to the tropomyosin on fixed L3, but not on mf. Such a discrepancy can be explained with the ability of filarial parasites to eliminate or deteriorate antibody binding and/or cell adherence to the cuticle. For instance, studies have shown that L3 have a turn-over of cuticle components and can presumably cast off bound antibodies, creating the situation in which macrophages or other effector cells would not be able to bind targeted antibodies (Apfel et al., 1992).

A. viteae tropomyosin not only induces B cell responses, but is also capable of inducing T cell responses. Results show that both forms of tropomyosin used have T cell stimulatory capacity as spleen cells of immunized mice, upon restimulation with different tropomyosins, mounted a proliferative response that was 3 to 5 fold higher than the control. Synthetic peptides comprising putative T cell epitopes of *A. viteae* tropomyosin were also able to restimulate splenocytes from immunized mice. Additionally, as shown by the algorithms of Rammensee et al. (1999) *A. viteae* tropomyosin can comprise up to 34 different T cell epitopes when examined against two different mouse MHC haplotypes *H2-Ak* and *H2-Ek*. This data are in line with the literature observations. As shown for shrimp tropomyosin, splenocytes of immunized mice were restimulated with the full length molecule and a synthetic peptide, spanning aa 261-281 at the C-terminal end of the protein. The proliferation induced by the synthetic peptide was comparable with the effect obtained by the complete protein, revealing strong T cell epitope(s) in the C-terminus (Subba Rao et al., 1998). Interestingly, administration of that peptide along with a Th1 inducing adjuvant (FCA) to mice immunized with native tropomyosin emulsified in alum, led to significant down regulation of tropomyosin specific IgG and IgE. Such a shift

can be explained by a modulation effect potentially promoted by alteration in cytokine profiles or activation of allergen-specific regulatory T cells (Fellrath et al., 2003; Unger et al., 2003).

Although there are only scarce data in the literature about immunological mechanisms underlying the effect of tropomyosin vaccination, the literature describes a helminth antigen with properties very similar to tropomyosin. Paramyosin of the blood flukes *S. mansoni* and *S. japonicum* is a prominent vaccine candidate antigen (McManus et al., 1998; Yang, 2000). Studies in a murine model of schistosomiasis revealed that activated macrophages efficiently kill schistosome larvae in paramyosin-immunized mice (Pearce et al., 1988). It was described that the activation occurs through IFN-γ produced by antigen specific T cells that are triggered by paramyosin released during early infection (James et al., 1982). Mice vaccinated against *S. mansoni* were best protected when native paramyosin was intradermally injected together with BCG (bacillus Calmette-Guerin), an immunization scheme driving immune responses towards Th1 responses (Lanar et al., 1986; Murray et al., 1996). Paramyosin has certain traits common with tropomyosin, both being muscle proteins with a coiled-coil structure and containing repetitive elements (Cohen et al., 1990). Recent studies have shown that paramyosin of mites and the parasitic nematode *Anisakis simplex* are potent allergens (Perez-Perez et al., 2000; Fischer et al., 2003; Lee et al., 2004). These striking parallels suggest that similar immune effector mechanisms could be operative in animals immunized with tropomyosin. This study showed that *A. viteae*-tropomyosin is a T cell antigen in mice which fact, bearing in mind previously cited schstosoma research, allows to hypothesize that macrophages could be possible effector cells killing infective larvae.

3.2. Allergenicity of A. viteae tropomyosin

This study showed that tropomyosin of the rodent filarial nematode *A. viteae* is allergenic in natural infections (Hartmann et al., 2006). Sera of infected jirds were used to sensitize rat basophil leukemia (RBL) cells, which degranulated upon stimulation with native and recombinant *A. viteae* tropomyosin, showing that the infection induced specific and reactive anti-tropomyosin IgE. Due to structural constraints that are essential for its primary function, tropomyosin has a repetitive structure, implying repetition of similar

epitopes. Such repeated cross-reactive domains can simultaneously cross-link several IgE molecules on the cell surface, leading to efficient aggregation of FcεR on the surface of sensitized basophils and mast cells (Turner and Kinet, 1999; Bruhns et al., 2005). Along this line, the high allergenicity of tropomyosin is partly due to a repetition of similar IgE-recognized epitopes within the molecule.

Moreover, *A. viteae* tropomyosin showed cross-reactivity with several different tropomyosins cloned from various nematodes and a shrimp tropomyosin. Although sera from rAvTropo immunized mice were used, high levels of mediator were released by cells in presence of rOvTropo, tropomyosin of *H. polygyrus* and tropomyosin from the free living *C. elegans.* Finally, cross-reactivity with shrimp tropomyosin was observed.

The extension and importance of cross-reactivity was shown, when IgE reactivity to shrimp occurred in unexposed individuals like orthodox Jews, subjects strictly observing Jewish tradition which regards seafood as a non-Kosher (Fernandes et al. 2003). Based on inhibition experiments house dust mite or cockroach-allergic subjects showed significant IgE Ab reactivity to the major shrimp allergen Pen a 1 due to cross-reacting tropomyosins of house dust mite and cockroach.

Results of this work are in line with studies that revealed massive cross-reactivity among tropomyosins of crustaceans, mites and cockroaches which also extends to nematode tropomyosin (Witteman et al., 1994; Martinez et al. 1997). Indeed, incubation of human onchocerciasis sera with shrimp tropomyosin could completely inhibit binding of specific antibodies to tropomyosin of the filarial nematode *O. volvulus* (Jenkins et al., 1998). Moreover, tropomyosins from shrimp, cockroach or house dust mite inhibited binding of specific IgE from patient sera to tropomyosin of *A. simplex*, a parasitic nematode sporadically transmitted by infected fish and known as “sushi worm” (Asturias et al., 2000). My data shows that inhibition between closely related species (competition of epitopes) was greater between closely related nematodes, but also distant related species like shrimp and *A. viteae* were cross-reactive and IgE specific to *A. viteae* tropomyosin was partially inhibited by the shrimp tropomyosin. This is noteworthy given 63% identity between two proteins. In the RBL system used here a difference in allergenic potential between nematode and shrimp tropomyosins varied between 2 to 100 fold depending from the dose used.

Interestingly, vertebrate tropomyosins were reported to be non-allergenic (Ayuso et al., 1999), and experiments presented in this work based on RBL degranulation assay with chicken and porcine tropomyosin corroborate these data as in none of three experiments vertebrate tropomyosins were cross-reactive, nor inhibited functional IgE. Although the amino acid sequence identity between invertebrate and vertebrate tropomyosins is relatively high, ranging from 51% to 58% (Reese et al. 1999), vertebrate tropomyosins do not bind IgE from shrimp-allergic subjects (Ayuso et al., 1999). Reasons for this lack of allergenicity are not entirely clear, however it is possible that tropomyosins of invertebrates might contain molecular signatures that stimulate the immune system of vertebrates in a particular way, leading to development of allergic responses (Saarne et al., 2003). Alternatively, due to the high homology among vertebrate tropomyosins, potential IgE epitopes might not be recognized as foreign due to phenomena of immunological tolerance.

A. viteae tropomyosin was shown here to be a potent allergen and yet a good vaccine candidate. It is believed that mechanisms leading to allergic responses against proteins are default pathways that function in the absence of strong Th1-inducing stimuli. The allergenicity may be, in addition, markedly influenced by the way of uptake a protein by APCs, and by the efficiency of presentation to T cells as well as by co-stimulatory molecules and other factors (Aalberse, 2000). However, if potential allergens are applied together with a Th1-inducing adjuvant, they will induce other types of responses. Thus, allergens can have potentially surprising properties, when applied under unusual conditions. As revealed by this study *A. viteae* tropomyosin can be an example of such a protein, being protective under particular conditions.

My experiments showed that there are numerous B cell epitopes within the aa sequence of *A. viteae* tropomyosin. The results of analysis with jird and BALB/c sera showed 13 functional IgG epitopes and 11 epitopes for IgE. IgG epitopes were spread along the whole tropomyosin molecule with the immunodominant epitopes localized in the center and on the C-terminus of the molecule. Sera from naturally infected animals recognized 6 IgE epitopes. However, sera from immunized animals showed presence of 11 distinct epitopes. 8 IgE epitopes colocalized completely or partially with IgG epitopes.

Interestingly, positions of most IgE epitopes characterized on *A. viteae* tropomyosin correspond very well with IgE immunodominant regions mapped on shrimp tropomyosin by screening of peptide libraries with sera from allergic patients (Ayuso et al., 2002a).

So far, there were several reports published on IgE-binding sites of shrimp, insect or arachnid tropomyosin, identified by screening of synthetic peptides libraries or chemical cleavage (reviewed by Reese et al., 1999). Authors identified various regions consisting of B cell epitopes and focused strongly on characterization of putative IgE regions. The most detailed study with allergic patient sera showed that 5 immunodominant IgE regions and additional 22 minor IgE binding regions (Ayuso et al., 2002a) are located on shrimp tropomyosin. 5 nominated regions located between aa 43-57, 85-105, 133-148, 187-202 and 247-284 appeared to be very similar in arthropods species and their existence was shown to account for most of the IgE cross-reactivity among shrimp, cockroach and house dust mite tropomyosins (Ayuso et al., 2002b; Saarne et al., 2003). It is interesting that the sequence homology extends also to parasitic nematodes like *A. viteae, O. volvulus, T. colubriformis, A. suum* and *T. spiralis*. However, my study shows that immunodominant IgE epitopes of *A. viteae* tropomyosin are located in different positions. A study of Ayuso et al., (2002a) showed that five major IgE-binding regions of shrimp tropomyosin contain an aa sequence characterized by a concentration of negatively charged aa, forming the LEXXL motif, where L is leucine and X is usually an acidic aa such as glutamic acid (E) or asparatic acid (D). Interestingly, these motifs always occupy positions *d*, *e*, *f*, *g and a* in the heptameric repeats. Except for position *f*, they represent domains of interactions between two polypeptide chains of tropomyosin, which in native tropomyosin are probably unexposed residues with side chains hidden within the coiled coil (Greenfield et al., 1995). Binding of IgE to these epitopes would require the exposure of the motifs, either by high flexibility of the tropomyosin dimer, by denaturation, or by degradation.

The alignment of protein sequences shows that such motifs are also present in the *A. viteae* tropomyosin sequence. Yet, in contrast to the cited results, my experiments with serum of naturally infected animals showed that only one region bound by IgE contained such motif. In addition, experiments with sera of animals immunized with *A. viteae* tropomyosin, aimed to pinpoint exact IgE epitopes utilizing short 10-mer peptides,

showed that none of found IgE epitopes contained a full LEXXL motif. The difference might be owned to several reasons. Firstly, there is 63% identity between the sequences of two proteins. This level of homology might be enough for a creation of a similar α-helical structure, yet too low for similarly constituted and located immunodominat IgE epitopes. It is possible that due to evolutionary distance between crustaceans and nematodes some differences in tropomyosin aa sequence influenced the structure of IgE inducing epitopes and their localization. Thus, IgE inducing regions do not necessarily bear such a core motif. This observation is augmented by the fact that in RBL experiments *A. viteae* tropomyosin was only partially cross-reacting with shrimp tropomyosin. Secondly, it has to be noted that two studies were done in different systems with peptides of various length and with use of different sera, which perhaps can not be directly compared. A more recent study on shrimp tropomyosin showed that out of 8 immunodominant individual epitopes (shortest peptides with the maximal IgE reactivity) that fell into 5 IgE reactive regions, only 3 contain a LEXXL motif (Reese et al., 2005).

Antibody binding sites are thought to be dependent on the conformation of the allergen, and epitope identification using short overlapping peptides has been criticized in that this strategy does not produce meaningful results. However, this strategy has been used to identify major epitopes of peanut allergens (Stanley et al., 1997, Shin et al., 1998, Rabjohn et al., 1999), and studies of milk and peanut allergens (Beyer et al., 2003, Cocco et al., 2003) suggested associations between IgE Ab reactivities to linear peptides and severity of the disease. Nonetheless, as antibodies mostly recognize conformational structures on the antigen surface, it is likely that epitope-mapping studies relying on synthetic peptides detect only a fraction of epitopes (Yi et al., 2002). Therefore, additional IgE epitopes of tropomyosin may be composed of side chains of aa in residues *b*, *c* and *f* and to a lesser extent *e* and *g* positioned in different locations on tropomyosin heptapeptide turns. An example could be paramyosin (UNC-15) of *C. elegans,* where mapping and analysis of B cell epitopes showed that hydrophilic aa residues create the core of the B cell epitopes (Cooper et al., 1997).

Limited results of the substitutional analysis of a single aa within the epitopes of *A. viteae* tropomyosin showed that in majority of cases it leads to decrease or even total

abrogation of antibody binding. In some cases however, replacing of a single aa residue with a different aa led to enhanced binding of antibody or was neutral and had no negative or positive effect. It seems that for both IgG and IgE epitopes the effect of substitution was related to the nature of the substituted aa and position occupied in heptapeptide. Aa in positions *e*, *f*, *g* were seldom replaced without deterioration or total loss of binding. On the other hand, substituting aa that positively influenced binding in IgG epitopes were F, H, I, K or R, while for IgE epitopes substitutions with K, L, R or F enhanced the binding of IgE. These amino acids could replace many other aa in epitopes residues. In contrast, aa residues that occurred to be the most important for the binding to the mutant peptides were R and K, since their replacement in majority of cases led to the abrogation or reduction of the antibody binding. The analysis showed that the amino acids important for antibody binding belong to the group of nonpolar hydrophobic (F, I) or basic positively charged (R, K, H) aa. Since lysine and arginine were critical for binding to the mutant peptides it is possible that their charge and long side chains contributed essentially to the epitope formation.

It seems that recognition of an epitope by the antibody is associated with the key positions within its sequence influencing creation of the epitope. Apparent increase in IgG or IgE binding may be due to acquiring of additional structural futures attracting more antibodies with different sequence specificity. Along this thoughts, decrease of binding or total abrogation shows that some key positions were vital, especially *e*, *f*, *g* residues at the outside of the helix.

Results from this study are partially in line with results published on shrimp tropomyosin. Similarly, to the situation in *A. viteae* Ayuso et al., (2002b) reported presence of key amino acid residues within IgE binding regions. Substitutions of these aa were associated with the loss of IgE binding. Interestingly, most of the substitutions that led to the significant decrease of binding were located in the center of the region. Together this results suggests that the mutated synthetic peptides can be either more or less immunogenic than the native ones, giving us an interesting tool in our search for the more immunogenic antigens or their parts (superepitopes) or hypoallergenic epitopes useful in treatment of allergic disorders. The idea of reducing or abolishing the IgE Ab binding capacity and preserving T cell reactivity is currently discussed as a promising

new strategy for the treatment of allergies (Bannon et al. 2001, Campbell et al. 2000). Because shrimp tropomyosin is responsible for at least 75% of the shrimp-specific IgE Abs (Daul et al. 1994), the IgE Ab-binding capacity of Pen a 1 had to be reduced substantially if it is to be used as a therapeutic reagent. Experimental results of Reese et al., (2005) gave credibility to that idea by showing that the allergenic potency of VR9-1 mutant shrimp tropomyosin that, was substituted in 12 positions critical for IgE binding, could be reduced between 10 to 40 fold in functional assay, which translated into a reduction of allergenic potency of 90-98%.

3.3. mAb against A.viteae tropomyosin, their epitopes and specificity

This study introduces three new monoclonal antibody raised against *A. viteae* tropomyosin NR1, R21 and N11. The analysis of binding pattern by immunoblots revealed some distinct differences between them. NR1 and N11 well bound to tropomyosin from all developmental stages of the parasite, however R21 did not recognize tropomyosin from PBS extract of L3 and Mf. On the other hand R21 was binding the best to rAvTropo. Additionally, immunoblots showed that NR1 and R21 but not N11 were not binding to vertebrate tropomyosins (with the of R21 binding purified porcine tropomyosin). Interestingly, both NR1 and N11 bind tropomyosin in PBS extract of shrimp muscle, but not to the recombinant protein. Comparison with the mAb TM311 (Nicholson-Flynn et al., 1996) showed that all three mAb have much higher ability to bind invertebrate tropomyosin.

Mapping and characterization of the epitopes for these mAb showed that both NR1 and R21 bind to the C-terminal part of the molecule. Interestingly, target epitope for the TM311 is located on the N-terminus of the tropomyosin (Nicholson-Flynn et al., 1996). N11 did not bind to the recombinant peptides. Together with the experiments using metaperiodate treatment of nAvTropo and *A. viteae* soluble extract, where N11 binding was decreased after anti-glycan treatment, it shows that N11 could be directed against an epitope consisting of a posttranslational modification. Moreover, data obtained in western blots show that in the total antigen extracts of female or male worms occasionally there were more bands recognized by this mAb. Interestingly, more

recognized bands also appeared in extracts from *O. volvulus* and *Hymenolepis diminuta* suggesting that N11 might be targeted against a common modification. Interestingly, N11 antibody strongly reacts also with several bands from vertebrate muscle PBS extracts including a 41 kD band corresponding to tropomyosin. In contrast, vertebrate tropomyosins prepared with a standard protocol after Greaser and Gergely (1971) were not recognized, although they were native, hence should bear similar modifications as proteins in PBS extracts.

Alignment of the NR 1 and R21 binding regions sequences from *A. viteae* tropomyosin with invertebrate and vertebrate tropomyosins showed major substitutions in vertebrate sequences. These substitutions changed the chemical nature of a given amino acid residue within the epitope: polar>nonpolar, uncharged>acidic or acidic>uncharged. Such changes probably result in different epitope conformation which could explain why these antibodies are invertebrate tropomyosin specific. Interestingly, in vertebrate proteins, only two amino acids were substituted with the amino acid of the same chemical character. Such changes are more abundant within tropomyosins of invertebrate species that were taken into consideration in this study. This fact suggests that such substitutions did not affect antibody binding. Such approach can explain the promiscuity of NR1 and R21 monoclonal antibodies and their ability to bind to muscle form of tropomyosin from many different species within the invertebrates. Partially conserved sequence of the NR1 region (RTVSARLKEAETRAEFAERS) within invertebrate species naturally allows that antibody to have a broader spectrum of recognized species. In parallel, the sequence of epitope for R21, which is far less conserved even between closely related invertebrates, does not allow for wider recognition or high binding intensity.

NR1 and R21 employed in this study target the C-terminally located region on *A. viteae* tropomyosin. The C-terminal part of tropomyosin has been reported as a one of the major immunogenic regions in tropomyosin associated food and airway allergies in humans (Santos et al., 1999, Ayuso et al., 2002a). It is tempting to speculate that the antigenicity of C-terminal region might be a common feature for all invertebrate tropomyosins.

As shown *in vitro* purified NR1 antibody was functional and effectively promoted ADCC related killing and destruction of microfilariae by mice naïve peritoneal macrophages. In this respect, the NR1 performed similarly to serum of naturally infected jirds or serum of microfilariae immunized hamsters. In addition it was also used successfully in immunofluorescence experiment showing that also L3 have tropomyosin on the surface of the cuticle.

3.4. Cellular alteration and cytokine profile induced by A. viteae tropomyosin

This study showed the ability of *A. viteae* tropomyosin to induce an increase of $Gr1^+/CD11b^+$ cells in spleen and of $Gr1^+/CD11b^+/F4/80^+$ cells in the peritoneal cavity of immunized animals. It was also observed that native *A. viteae* tropomyosin could elicit the production of IL-4 and IL-10 by $Gr1^+/CD11b^+$ cell subpopulation, while rAvTropo stimulated production of IL-10.

High levels of IgG1, IgG3 and IgE antibody responses evoked by immunization of mice with rAvTropo and nAvTropo and a response strongly biased towards the Th2 phenotype create a situation similar to the effects of immunization with aluminium hydroxide (alum). These observations led to the assumption that *A. viteae* tropomyosin induce similar pathways to those initiated by alum injection. Jordan et al. (2004) showed that alum led to priming of B cells and to the accumulation of $Gr1^+/CD11b^+$ cells in the spleen that produced IL-4 and were required for priming and expansion of antigen specific B cells. Studies on *A. viteae* tropomyosin showed that only nAvTropo induced statistically significant IL-4 production in assays with splenocytes and spleen derived $Gr1^+/CD11b^+$ cells from immunized mice. Moreover, the observed level of cells in this subpopulation was very similar to that from alum immunized animals. Interestingly, although rAvTropo did not induce detectable IL-4 production, it promoted production of $Gr1^+/CD11b^+$ in spleen. These data suggest that native *A. viteae* tropomyosin has some intrinsic capabilities similar to alum, however further evaluation would be needed to address this issue, especially if adjuvant properties were considered.

Interestingly, adjuvant features were already described for excretory-secretory proteins (NES) of *Nippostrongylus brasiliensis*, a gut dwelling nematode parasitizing rodents (Holland et al., 2000). Administration of NES to mice resulted in high IL-4 production and

elevated IgE levels. Even when applied with CFA (Complete Freund's adjuvant), which normally favors Th1 responses, NES vaccination led to profound Th2 type response. Another example could be *S. mansoni* egg antigens that upon exposure bias cytokine responses towards Th2, even in presence of unrelated Th1 favoring antigens, or infections that normally develop Th1-dominant response or of Th1-mediated autoimmune disease (Actor et al., 1993; Curry et al., 1995; Cooke et al., 1999).

In vitro studies with $Gr1^+/CD11b^+$ splenocytes showed that both forms of *A. viteae* tropomyosin are able to induce specific production of IL-10. This finding might have important implications for the paradoxical observations that the prevalence of allergic diseases in patients with helminthiasis is lower as compared to uninfected individuals, despite their long-term exposure to helminth allergens and the observation that a potent allergen in a natural infection might not evoke a full allergic reaction. However, it has been shown in animal models as well as in cross sectional and in interventional studies in human populations that helminth infections are associated with a decreased prevalence of allergic disease caused by environmental allergens, e. g. house dust mite allergens (van den Biggelaar et al., 2004; Maizels and Yazdanbakhsh 2003; Yazdanbakhsh and Wahyuni, 2005).

The literature provides several mechanisms described for various parasite-host systems, which might cooperate or act in parallel in a given host. First, it has been demonstrated that chronic infections with the murine intestinal nematode *H. polygyrus* induce IL-10 and TGF-ß-producing regulatory T cells, which suppress allergic responses against the model allergen ovalbumin (OVA, Wilson et al., 2005). Upregulation of IL-10 was also reported in infections with *A. suum* and *Nippostrongylus brasiliensis* (van den Biggelaar et al., 2000; Wohlleben et al., 2004; van den Biggelaar et al., 2004; Schopf et al., 2005).

Second, high levels of antibodies other than IgE can interfere with efficient mast cell degranulation. "Blocking antibodies" of the IgG4 subclass might compete with specific IgE for epitopes, thus reducing the chances that mast cells and basophiles degranulate after contact with antigen (Turner et al., 2005). Furthermore, nematode infections have been shown to upregulate nonspecific IgE, which leads to a dilution of specific anti-allergen IgEs and reduces the ability of mast cells and basophiles to degranulate upon

antigen contact (Lynch et al., 1998). Third, released nematode products might inhibit the chemoattraction and activation of effector cells, e. g. by degrading eotaxin, a chemoattractant and stimulator of eosinophils. Impaired cell migration to the site of basophile/mast cell degranulation would reduce eosinophilic inflammation induced by parasite stages, but also by environmental antigens (Culley et al., 2000).

However, other single components of parasitic organisms such as Schistosome oligosaccharide Lacto-N-neotetraose or Lacto-N-fucopentanose III expanded a cell subpopulation expressing Gr1/CD11b/F4/80 within the peritoneum and that these cells along with low levels of proinflamatory cytokines, produced high levels of IL-10 and TGF-β. Moreover these cells were suppressive in direct contact with $CD4^+$ T cells and this effect was dependent on INF-γ and NO (Terrazas et al., 2001; Atochina et al., 2001). Another study found that intraperitoneal injections of intact glycans from *Taenia crassiceps* metacestode recruited a population of $Gr1^+/CD11b^+$ cells which suppressed naïve T cell proliferation in a cell-contact dependent manner after adoptive transfer (Gomez-Garcia et al., 2005).

In addition, research on *Toxoplasma gondii* showed that a macrophage-like cell population also characterized by expression of Gr1, CD11b and F4/80 cell surface molecules was recruited to the peritoneal cavity 4 days after infection. These cells produced reactive nitrogen intermediates, IL-12p40 and were shown to contribute to the parasite control during infection (Mordue and Sibley, 2003). Voisin et al. (2004) showed that $Gr1^+/CD11b^+$ cells were present in lungs of mice infected orally with *T. gondii.* These $Gr1^+$ macrophage-like cells induced production of INF-γ, NO and IL-10. Finally, a study of *Trypanosoma cruzi* infection in mice showed that unresponsiveness of splenocytes to parasite antigens was associated with a huge increase in $GR1^+/CD11^+$ cells producing NO. Purified $Gr1^+/CD11b^+$ cells were expressing iNOS upon INF-γ treatment and were able to suppress T cell proliferation (Goni et al., 2002). Finally, $Gr1^+/CD11b^+$ cells were termed natural suppressor cells expanding in animals injected with viruses or mice harboring tumors (Bronte et al. 1998; Kusmartsev et al. 2000). Results from experiments performed after immunization with rAvTropo are in line with information cited above.

18 hours after immunization an increase of a distinct $Gr1^+/CD11b^+/F4/80^+$ cell population was observed in the peritoneal cavity of mice in comparison with naïve animals and

control protein recipients. The treatment with rAvTropo and nAvTropo resulted in a 5 and 4 fold increase in the $Gr1^+/CD11b^+$ cells, respectively. $Gr1^+/CD11b^+/F4/80^+$ population reached 7.7% of a total number of cells, while $Gr1^+/CD11b^+$ represented 25.1 % of cells. An additional approach to distinguish different cell populations by cell spin showed that animals treated with rAvTropo showed a strong increase in neutrophils (up to 10 fold), eosinophils (4 fold) and basophiles (3 fold) and a decrease in lymphocyte number (2 fold) as compared to the naïve animals. The macrophage population remained at the similar level as in control animals. In addition, purified $Gr1^+/CD11b^+$ cells from peritoneal exudates of tropomyosin immunized animals showed production of IL-10.

Interestingly, after implantation of adult *B. malayi* an expansion of $Gr1^+/CD11b^+$ suppressor cells in the peritoneum was also shown. In these experiments the interaction of suppressive PECs with T cells resulted in Th2 cytokine production (MacDonald et al. 1998, Loke et al. 2000).

Taken together it is likely that these $Gr1^+/CD11b^+/F4/80^+$ cells promoted by *A. viteae* tropomyosin treatment could be suppressor cells that skew T helper responses in the way of filarial favorable Th2 and in addition induce production of IL-10. However, further evidence is needed to characterize the nature and pathways of processes observed here. Thus, this work characterizes *A. viteae* tropomyosin as a defined helminth protein that induces IL-10. Although helminthiasis patients generally display high levels of IL-4, IgE and eosinophilia, they are less likely to develop clinical allergy. The findings described here suggest that this obvious contradiction may be explained by the intrinsic ability of helminth proteins such as tropomyosin to induce IL-10 expression. The resulting permanent stimulation of anti-inflammatory responses by tropomyosin may help to protect the host from developing allergies by down-regulating certain immunologic responses as also described by Wilson and Maizels (2004).

3.5. Outlook

Investigations of the molecular and immunological properties of filarial nematodes antigens along with the mechanisms of parasitism are essential to develop novel therapeutic and preventive strategies against filariasis. In this study *Acanthocheilonema viteae* tropomyosin was characterized.

It was determined that *A. viteae* tropomyosin is an immunodominant antigen that elicits strong specific antibody responses and skews the host immunological environment towards the Th2 profile. The fact that tropomyosin elicits specific and functional IgE along with observations from subsequent cross-reactivity and inhibition functional assays allows to assume that parasite tropomyosin is an allergen. Immunodominant properties of *A. viteae* tropomyosin are probably due to the ability to elicit IL-4 and prime B cell for production of antibodies by the similar mechanisms as observed for alum. However, *A. viteae* tropomyosin induces also production of IL-10 by the $Gr1^+/CD11b^+$ cells what could prevent the allergenic responses against this antigen. This interesting issue would have to be addressed in future studies, especially in respect to the receptor and mechanisms of induction. It seems interesting that parasites could render inactive an allergen that is otherwise an essential structural protein or modify it so, that it serves to suppress unwanted reactions.

Perhaps that is why *A. viteae* tropomyosin used in vaccination experiments induced moderate protection, but only when applied in a scheme that droved immunological responses into the Th1 direction. This indicates that parasite tropomyosin can be an interesting component for future studies on more complex vaccines or perhaps a therapeutic reagent useful to prevent or diminish allergic reactions against similar antigens (like tropomyosin associated seafood and air allergies induced by invertebrates). Experiments in this direction would determine the importance and magnitude of the immunomodulatory effects of *A. viteae* tropomyosin.

Taken together results of this study demonstrate that tropomyosin plays an important role for the parasitism of *A. viteae* and therefore is an interesting antigen that could be considered as an intervention target.

4. Methods

4.1. Parasitological methods

4.1.1. Laboratory animals

Six- to eight-week old male BALB/c or C57/BL6 mice were used in this study for immunization and analysis of cells. BALB/c were bread in Department's own animals facility while C57/BL6 mice were purchased from (Berlin) and were maintained in a pathogen free environment at Department of Molecular Parasitology animal facility in accordance with institutional guidelines.

4.1.2. Maintenance of the life cycle of Acanthocheilonema viteae

The life cycle of *A. viteae* was maintained essentially as described by Lucius and Textor (1995). Briefly, the infective L3 stages of *A. viteae* were obtained from infected *Ornithodoros moubata* ticks and used to infect *Meriones unguiculatus* subcutaneously. The L3s develop to the L4 and subsequently to the adult male and female stages. The female worms release microfilariae into the peripheral blood. The peripheral blood of infected *Meriones* was used to infect ticks where the microfilariae develop to the L2 and subsequently to the infective L3 stages.

4.1.3. Immunization experiments

The protective potential of *A. viteae* tropomyosin was evaluated in the *Meriones/A. viteae* natural host-parasite system. Eight to ten weeks old *Meriones* were anaesthetized with ketamin:xylazin:normal saline (1:1:8) mixture and immunized. Recombinant *A. viteae* tropomyosin, peptide or plasmid containing *A. viteae* tropomyosin DNA were used as vaccines For DNA immunization studies, 50 μg of plasmid per animal was used intramuscular in the thigh muscles. For protein immunization, 25 μg of protein per animal was used subcutaneously. The animals were immunised three times with two weeks intervals, after which they were challenged with 70 freshly isolated *A. viteae* L3s by injecting subcutaneously in the neck region. Microfilarial load was verified at weeks 8 and 11 post infection (p.i.), and the animals were sacrificed and dissected at 11 weeks p.i. for isolation of adult worms and determination of vaccination protective potential.

Different adjuvants like alum (aluminium hydroxide 2% gel), STP (Sqalane, Tween, Pleuronic) and AdjuPhos (aluminium phosphate 2% solution) were used for immunisation.

4.1.4. Antigen injection in $Gr1^+/CD11b^+$ experiments

Two different forms of tropomyosin (nAvTropo, rAvTropo) and a recombinant control antigen rDHFR were used for this study. 25 µg of purified proteins diluted in in 250 µl of endotoxin-free water were administered intraperitoneally per animal. 144 or 18 hours after injection depending on the experiment animals were sacrificed by terminal inhalation of CH_4 and subsequent cervical dislocation.

Splenocytes were obtained 6 days after administration of antigens by rapture of the spleen and pressing it through a fine metal mesh. Cells were washed twice in ice-cold PBS and red blood cels (RBC) lysed by hypotonic shock with ammonium chloride. Viable cells (trypan bleue exclusion) were counted and adjusted to 1×10^6 cells / ml.

PECs were isolated 18 hours after the injection by peritoneal lavage with 10 ml of ice-cold RPMI 1640. Cells were washed twice and RBC were lysed. Viable cells were counted and adjusted same like splenocytes. Both types of cells were analyzed for cell markers and cytokine production.

4.1.5. Quantification of microfilarial load in blood of jirds

Infected *Meriones* were anaesthesized with ketamin:xylazin:normal saline (1:1:8) and bled from the retro-orbital sinus using a heparinised glass capillary tube. The blood (44.7 µl) was mixed with 100 µl Teepol (10 % in H_2O) and the microfilarial load was estimated using a Fuchs-Rosenthal counting chamber.

4.1.6. Isolation of adult A. viteae from M. unguiculatus

Meriones were anaesthetized and fully bled from the retro-orbital sinus. Following dissection of the jirds, adult *A. viteae* were isolated from the muscles and subcutaneous tissues, the inguinal and subscapular regions and some times from the thoracic chamber. Animal carcasses were then incubated in normal saline (0.9 % NaCl) overnight to allow the rest of the worms to wander out. The female and male worms were collected

separately in complete RPMI 1640 medium supplemented with pencillin and streptomycin (final concentration of 1unit / ml), L-glutamate (final concentration of 2 mM) and fatal calf serum (final conc. of 10%).

4.1.7. Isolation of L3 stages from the vector Ornithodoros moubata

L3s of *A. viteae* were isolated from infected *O. moubata*. The ticks were cut medially and briefly rinsed in a Petri dish with incomplete RPMI (iRPMI) to remove rests of blood meal and loose tissue. Ticks were incubated in warm iRPMI solution for 30 min for the L3s to migrate. The L3s were washed in iRPMI thoroughly for use in further experiments.

4.2. Cell culture methods

4.2.1. Maintenance of mammalian cells

COS7 (African green monkey kidney cell type) and HeLa cells (human epithelial cells from a fatal cervical carcinoma) were maintained in complete RPMI 1640 under standard tissue culture conditions at 37°C and with 5 % CO_2. When the culture flasks were confluent, the cells were washed with 1X PBS and treated with Trypsin/EDTA solution at 37°C for 2 min and scraped with cell scraper so that they were not adhered to the flask bottoms. The cells were centrifuged and washed with complete RPMI to remove trypsin and a portion of this was resuspended in complete RPMI to be passaged.

RBL (rat basophile leukaemia) cells were maintained in a similar way using complete MEM-EARY medium and no Trypsin/EDTA solution was used.

X63.Ag8 myeloma were cultured in RPMI 1640 supplemented with 20 % of FCS.

4.2.2. Preparation of stocks

The mammalian cells were suspended in complete RPMI to obtain a concentration of 10^7 cells / ml. To this, sterile dimethyl sulfoxide (DMSO) was added to a final concentration of 8% and frozen slowly in a styrofoam box at -80°C and later transferred to liquid N_2 for long term storage.

4.2.3. Transfection of COS7 and HeLa cells

Expression of *A. viteae* tropomyosin was analyzed *in vitro* by transfection of COS7 cells with pcDNA/AvTropo. 2 x 10^5 COS 7 cells / six well plate were cultured in 2 ml Dulbecco's MEM (DMEM) medium supplemented with 10% FCS, 100 U / ml penicillin, 100µg / ml streptomycin and L-glutamine. Cells grown to 50 – 70% confluence were transfected by addition of a solution of 1 µg plasmid pcDNA/AvTropo or pcDNA 3.1^+ in 3 µl of FuGENE 6 and 97 µl of serum free DMEM medium. After 30 minutes of incubation the mixture was evenly, drop-wise, added to the cell culture wells and incubated for 48 h after a gentle mix. To determine transfection efficiency, cells were transfected in the same way with a control plasmid pEGFP-N1 encoding the EGFP protein and monitored for fluorescence under UV-light. The transcription of AvTropo sequence was analyzed by RT-PCR using specific *A. viteae* tropomyosin primers.

4.2.4. Raising the monoclonal antibody

Two groups of three BALB/c mice were immunized subcutaneously 3 times in 10-14 days intervals with 25 µg of rAvTropo or eAvTropo emulsified 1 : 1 v / v in alum. 48 h before fusion best responding animals (checked by ELISA) were boosted intravenously (iv) with 25 µg of antigen in PBS. These animals were later dissected and its splenocytes were washed and suspended in 4°C serum free RPMI. Similarly, naïve mice thymus feeder cells were prepared. Spleen cells were mixed with myeloma X63-Ag8.653 cells in proportion 3:1 and washed. Supernatant was carefully removed and cells were incubate for 3 minutes in 37°C dissolving the pellet. 1 ml of warm PEG 1500 was added drop wise with constantly mixing. Mixture was incubate at 37°C for additional 45 seconds without mixing and serum free medium was added in step proportions 1 ml every 30 seconds up to 50 ml. Fused cells were centrifuged, the pellet was carefully dissolved and resuspended in 450 ml HAT medium. At this point the feeder cells were added and the mixture was plated in flat-bottom 96 well plates at 200 µl per well. Cells were incubated at 37°C and 5% CO_2 for 7-9 days at which time first clones were visible.

4.2.5. Subcloning

12-14 days after fusion clones were screened by ELISA and positive ones were transferred into 48 well cell plates with 500 µl of fresh HAT. They were allowed to expand till 1/4-1/3 of bottom was covered by cells and screened once more. Subsequently best positive clones were transferred to 24 well cell plates in 1 ml of HT medium and after 2-3 days of growth they were subcloned by transferring 2 µl of cells into 20 ml of HT medium and plating 200 µl into 96-well plates. 1 to 10000 dilution allowed the expantion of single cells in wells. Plates were incubated at 37°C and 5% CO_2 for 7 days and growing clones were screened for production of specific antibodies in ELISA. Positive clones were a subject to a second and a third subclonings and screenings. Positive clones were grown in complete RPMI and eventually transferred into culture flasks.

4.2.6. Rat Basophil Leukemia (RBL) cell mediator release assay

The cell line RBL-2H3 was maintained in Eagle's MEM with 10% fetal calf serum. The cells were passively sensitized by incubation for 1h with jirds sera in a final dilution of 1:50 in Eagle's medium. For cross-linking, sensitized RBL cells were incubated in a volume of 100 µl with a different dilutions of antigens or total worm extract in Tyrode's buffer (TB) for 1 h in a humidified atmosphere at 37°C. Controls without antigens were run to measure spontaneous release of the mediator β-hexosaminidase. Total release was measured by lysis of cells with TB containing 1% Triton X-100. Release of β-hexosaminidase was detected by colorimetric quantification of the enzymatic activity on p-Nitrophenyl N-acetyl β-D-glucosaminide used as substrate. Results were expressed as percentage of the total release after deduction of spontaneous release. The assays were performed in triplicates.

4.2.7. T cells proliferation assay

Cultures of spleen cells of mice were set up in triplicates in 96-well round bottom microtiter plates in a 200 µl of RPMI 1640 supplemented with 10% FCS. Cultures were kept in a humidified atmosphere at 37 °C containing 5% CO_2 for 5 days. Antigen final concentration was 2 µg per well. Negative controls of cells alone in medium and positive

controls of cells incubated with concanavalin (ConA, 2 µg/ml) were included in each assay. For the final 18 h, 0.5 µCi of ^{3}H-labeled thymidine was added to each well. Incorporated radioactivity was quantified by scintillation counting with a Trilux 1450 counter. Results were expressed as a mean of triplicates in net counts per minute (cpm). Net cpm were cpm of stimulated cultures minus cpm of unstimulated control cultures.

4.3. Immunochemical and immunological methods

4.3.1. Bleeding of animals for production of sera

Mice and jirds were bled from the retro-orbital sinus using a microhaematocrit capillary, and the blood was stored at 4°C overnight. Blood samples were centrifuged at 10,000 rpm at 25°C for 20 min to separate the sera from blood clots. Sera were stored in 100 µl aliquots at -20°C.

4.3.2. Western blot

Proteins separated by SDS-PAGE were immobilised onto nitrocellulose membranes by Semi-Dry electrophoretic transfer (Towbin et al., 1979). Nitrocellulose and SDS-gel were sandwitch between 4 Whatmann paper sheets cut to the size of the gel and soaked in Semi-Dry transfer buffer. It was placed between the electrodes so that the gel with the protein was towards the negatively charged cathode and the nitrocellulose membrane towards the positively charged anode. A constant current of 80 mA was then applied across the cassette at room temperature for 45 min. Later the nitrocellulose membranes with immobilised proteins was blocked by incubation for 45 min in 5% skimmed milk powder in TBS or 3% BSA/TBS for 1h. Washed membranes were incubated with serum or a primary antibody for 1 h at room temperature followed by three 5 min washes in TBST (TBS, 0.02% Tween 20, pH 7.4). The membrane was then incubated with a secondary conjugate antibody for 1 h followed by washes as above. To detect antibody a substrate for the alkaline phosphatase enzyme conjugate, 5-Brom-4-chlor-3-indolylphosphate (BCIP) and tetrazolium chloride (NBT) in alkaline phosphatase buffer were used. All antibodies were diluted in the blocking solution.

4.3.3. Detection of specific antibody in sera of M. unguiculatus and BALB/c mice

Round or flat bottom 96-well plates were coated with 100 ng/well of a coating antigen in carbonate buffer (pH 9.6) and incubated overnight at 4°C. After washing three times with PBST (PBS, 0.02% Tween 20, pH 7.4) the wells were blocked for 30 min with 100 µl 3% BSA/PBS at 37°C. After subsequent washing with PBST, sera were added in a dilution ratio of 1:80 in blocking solution and incubated for 2 h at 37°C. Subsequently, the plates were washed again with PBST and incubated with the secondary antibodies: goat anti-mouse IgG or IgM or rabbit anti-mouse IgG1, IgG2a, IgG2b and IgG3 conjugated to alkaline phosphatase in a dilution ratio of 1:3000 in 3% BSA/PBS. After 1 h incubation at 37°C the plates were washed again and the substrate (5 mg p-nitrophenyl phosphate disodium) dissolved in 10 ml carbonate buffer with 1 mM $MgCl_2$ was added. The reaction was stopped with 0.5 N NaOH and the absorbance at 405 nm with the additional reference filter at 630 nm was measured. All assays were performed in triplicates.

4.3.4. Immunostaining of A. viteae larvae

The *A. viteae* L3 and mf were stained to analyse the expression of tropomyosin on the cuticle. The larvae were fixed by treating with 200 µl of 4% formaldehyde for 5 min followed by 5 min in cold methanol, washed thrice with 500 µl of PBST with 0.5% Tween 20. The blocking was done with 100 µl of 10% FCS for 30 min followed by incubation with 100 µl of 1:25 dilution of primary antibody (monoclonal anti-tropomyosin NR1) in 10% FCS for 2 h. Larvae were washed with 500 µl of PBST twice and incubated with FITC-labelled anti-mouse secondary antibody diluted in a ratio of 1:500 in 10% FCS at 37°C in the dark. After subsequent triple washing with PBST were suspended in 50 µl of PBS and mounted on slides for microscopy.

4.3.5. Creation of the synthetic peptides libraries

For the identification of epitopes on A. viteae tropomyosin for both sera antibody and monoclonal antibody used in this study synthetic peptides libraries were used. Membranes of peptides (20 or 10 amino acid residues in length) representing the sequence of *A. vitaeae* tropomyosin with a shift of three amino acids were created. Peptides were synthesized on Whatman 50 membranes by semi-automated SPOT

Synthesizer as described in previous report (Kramer et al.,1999). The peptide arrays were generated on cellulose membranes that were modified with epibromohydrin and 4,7,10-trioxa-1,13-tridecanediamin for animo-functionalization (Ast et al., 1999). This type of membrane was used, since it is most stable during regeneration procedures.

4.3.6. Screening of synthetic peptide libraries

Detection of epitopes on within synthetic peptides libraries was performed using previously published protocols (Frank et al., 2002, Reinike et al., 2002) with small modifications. Membranes with peptide array were pre-incubated in TBST blocking buffer (+ 1 % sucrose) for 2 h. Subsequently the membranes were washed with TBS pH 7.4 and incubated with the prepared serum or mAb supernatant diluted 1:5 in TBST for 4 h in RT or overnight in 4°C. After washing three times for 8 min with TBST, appropriate secondary antibody, anti-mouse peroxidase-labeled IgM, IgG or IgE were added at a concentration of 1 µg / ml in TBST blocking buffer for 2 h in RT. The incubation was followed by washing 3 times for 8 min with TBST. Analysis and quantification of peptide bound antibodies and recording of the resulting spot patterns was carried out using a chemiluminescence substrate (soaking 2 min) and the Lumi-Imager™. The same procedure was used for control irrelevant mAb, negative sera and unspecific binding of conjugates. Successful removal of peptide-bound antibodies was confirmed by control incubations with secondary antibody alone before reusing the peptide array.

4.3.7. ELISA

Antibody subclasses in sera of vaccinated jirds or immunized mice were determined by ELISA. 96-well round-bottom plates were coated with 100 ng / well of rAvTropo in carbonate buffer (0.14 % NaCl, 0.3 % $NaHCO_3$), pH 9.6 and incubated overnight at 4°C. After washing 3 times with PBS, pH 7.4 / 0.025 % Tween 20 (PBST) the wells were blocked for 30 minutes with 100 µl 3% BSA / PBS at 37°C. After subsequent washing with PBST, individual sera were added in a dilution of 1 : 80 in 3 % BSA / PBS and incubated for 1 hour at 37°C. Subsequently, the plates were washed again with PBST and incubated with the secondary antibodies: goat anti-mouse IgG or IgM or rabbit anti-mouse IgG1, IgG2a, IgG2b and IgG3 conjugated to alkaline phosphatase in a dilution of

1: 3000 in 3 % BSA / PBS. After 1 hour of incubation at 37°C the plates were washed again and the substrate (5 mg p-Nitrophenyl Phosphate Disodium) dissolved in 10ml carbonate buffer with 1 mM $MgCl_2$ was added. The reaction was stopped with 0.5 M NaOH and the absorbance was measured at 405 nm with the additional reference filter at 630 nm.

4.3.8. IgE ELISA

For IgE ELISA, IgG depleated sera was used. 96-well flat-bottom plates were coated with 200 ng / well of nominated peptide in carbonate buffer pH 9.6 and incubated overnight at 4°C. After washing 3 times with PBST the wells were blocked for 30 minutes with 100 µl 5 % skimmed milk in PBS, pH 7.4 at 37°C. After subsequent washing 3 times with PBST, individual mice sera were added in a dilution of 1 : 5 in 3% BSA / PBS and incubated for 1 hour at 37°C. Subsequently, the plates were washed 3 times with PBST and incubated with the secondary antibodies rat anti-mouse IgE diluted 1 : 2000 in 3 % BSA / PBS. After 1 hour of incubation at 37°C the plates were washed 3 times and 50 µl of the substrate TMB (3,3',5,5'-tetramethylbenzidine) dissolved in 5 ml of distilled water and 5 ml of phosphate-citrate buffer pH 5.0 with addition of 2 µl of hydrogen peroxide were added. The reaction was stopped with 75 µl of 1 M sulfuric acid and the absorbance was measured at 450 nm with the additional reference filter at 630 nm.

4.3.9. Cytokine ELISA

For cytokine analysis all cell were cultured in complete RPMI 1640. Cells were adjusted to the concentration of 5 $x10^5$ / ml and plated in 24 well plates and maintained at 37ºC and 5 % CO_2 for 72 hours. Some cells were restimulated with antigens in concentration of 1 µg / ml. Supernatants were harvested, centrifuged and examined for production of INF-γ, IL-4 and IL-10 with BD Pharmingen OptEIA mouse-cytokine ELISA kits.

4.3.10. Flow cytometry FACS

Splenocytes and peritoneal exudates cells were blocked with mAbs against FcγR (CD16/CD32) and stained with FITC-conjugated anti-Mac-1 (CD11b), Cy5-conjugated anti-F4/80, biotin-conjugated anti-Gr1 (Ly-6G) (secondary antibody labeled with SA-

PeCy7). Antibodies were used in 1 : 1000, 1 : 500 or 1 : 250 dilution and incubated with cells for 15 minutes on ice in dark. After incubation cells were washed with ice-cold PBS / 1% BSA and suspended in 250 µl of PBS / 1% BSA for analysis.

Anti-Mac-1 were obtained from BD Pharmingen, anti- FcγR, anti-F4/80 and anti-Gr-1 were a gift from the German Arthritis Research Center in Berlin. Cytometric analysis was performed using FACS Calibur LSRII and live cells were electronically gated with forward and side scatter parameters. Later the results were analysed with FlowJo software.

4.3.11. Depletion of IgG antibody from sera of immunized mice and jirds

For depletion of IgG from the immune sera a modified method of Lerher at al., (2000) was used. To remove inhibiting IgG from sera, equal volumes of protein G beads (Dynal Biotech, Hamburg, Germany) and serum (diluted 1:10 or undiluted) were mixed and incubated at room temperature for 40 min. The samples were then placed next to a magnet to collect the beads at the wall of sample tube and the supernatant serum was removed. The bound IgG were eluted with elution buffer for 2 min (0.1 M sodium citrate, pH 2.8). Beads were regenerated by washing shortly two times with 0.1 M sodium phosphate (pH 6.0) and the process was repeated 3 times. This allowed depletion of approximately 70-80% of specific anti *A. viteae* tropomyosin IgG antibodies as investigated by ELISA. The serum volume was measured prior to and following absorption and it was shown that approximately 80% of original volume was recovered following all absorptions.

4.3.12. Deglycosylation of proteins on ELISA plate

Deglycosylation of proteins was performed on 96-well flat-bottom plates which were coated with 100 ng / well of nAvTropo or total worm extract in carbonate buffer (0.14 % NaCl, 0.3 % $NaHCO_3$), pH 9.6 and incubated overnight at 4°C. After standard washing 3 times with PBS, pH 7.4 / 0.025 % Tween 20 (PBST) the wells were additionally washed with 50 mM of Natrium acetate buffer, pH 4.5. Wells were incubated with 30 mM of Na-Metaperjodate in Na-acetate buffer for 1 hour at RT in dark. Subsequently, washing with Na-acetate was repeated and wells were incubated for 30 min with 50 mM of Na-

borhydride in PBS. After triple washing with PBST the plates were a subject to a standard ELISA.

4.4. Protein analytical methods

4.4.1. Preparation of soluble protein extracts

For preparation of soluble proteins extract from nematodes or tissue samples a method of PBS extraction was used. Worms or tissue was placed on ice, cut into fine pieces with scissors and suspended in PBS pH 7,4 with addition of proteinase inhibitors cocktail. Subsequently, sample suspension was homogenized in a glass homogenizer, and sonicated 4 times 30 sec. with 30 W. After sonication sample was centrifuged at 10,000 g for 15 minutes, to collect insoluble elements and debris. Supernatant containing tropomysins was transferred into a fresh conical tube and a whole protein concentration was estimated with a BCA test and sterilized by filtering. Aliquoted extracts were stored at –20° C until use.

4.4.2. Determination of protein concentration

Protein concentration was determined using the BCA kit. The basis of this assay is a biuret reaction: reduction of Cu^{2+} to Cu^{1+} by a peptide bond under alkaline conditions. Chelation of two molecules of bicinchoninic acid (BCA) with the cuprous ion (Cu^{1+}) produces a water soluble complex, whose solution has a deep purple colour and an absorbance at 562 nm. A linear standard curve was made with BSA concentrated between 2 and 0.05 mg and concentrations of the protein samples were determined using this standard curve.

4.4.3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were heated for 5 min at 100°C in sample buffer and applied at volumes of 10 - 30 µl to a 12 % slab gel cast in a mini-gel apparatus. The electrophoresis was performed at 20 mA with electrode buffer. The run was stopped when the bromophenol blue dye front reached the end of the gel. Protein bands were observed by staining the gel with Coomassie Brilliant Blue R250 and the gels were dried for a permanent record.

4.4.4. Maleylation of rAvTropomyosin

Recombinant *A. viteae* tropomyosin was maleylated in concentration of 1 mg / ml in 5 ml of carbonate buffer (pH 9) at 20°C. 5ml of protein solution was rapidly stirred and to avoid dilution 5 mg of a solid maleic anhydride was gradually added to the buffer. The pH was constantly monitored with a pH-meter and was maintained at the same range by titration with 5 M sodium hydroxide. After 10 minutes no more pH shift towards acidic was observed and the reaction was complete. Maleylated protein was then recovered by extensive dialysis against PBS (pH 7.4) at 4°C and loaded on SDS-PAGE gel. A small molecular weight value shift in line of maleylated form of rAvTropo was visible (ca. 3 kB) wile non-maleylated form run normally. Protein was stored in – 20 °C until further use.

4.4.5. Preparation of gel-eluted antigen

Native *A. viteae* tropomyosin (eAvTropo) was prepared by electroelution from SDS-PAGE-separated adult worm extracts. Adult female worms extracts were prepared in PBS, pH 7.4 by homogenization and subsequent sonication for 2 min and centrifugation (10 min, 12000 g). The position of the tropomyosin band was determined by Western Blot analysis of strips of the gel with an anti-*A. viteae* tropomyosin mAb and the protein was excised out of the SDS gel after copper staining. Subsequently, the protein was electro-eluted with a BioTrap BT 1000 kit. Eluted native tropomyosin was then dialyzed twice against PBS, pH 7.4.

4.5. Molecular biology methods

4.5.1. Preparation of DNA vaccine

The cDNA sequence of *A. viteae* tropomyosin was subcloned into the Eco RI / Apa I restriction sites of the mammalian expression vector pcDNA 3.1+. The recombinant plasmid pcDNA/AvTropo was transformed into competent *E. coli* JM109 and plasmid DNA from a 100 ml liquid culture was purified with NucleoBond Plasmid Maxi Kit according to the manufacturer's instructions. The recombinant plasmid DNA was resuspended in sterile millpore water and stored at –20°C.

4.5.2. Electrophoresis and detection of DNA on agarose gels

Agarose gel electrophoresis was used for the routine analysis of DNA. Agarose gels were cast in 1X Tris Acetate EDTA buffer containing 0.5 µg/ml ethidium bromide. The DNA samples (0.1 to 5 µg) were dissolved in DNA loading buffer and separated in agarose gels at 10 volts per cm. The concentration of the agarose gel was relative to the size of DNA fragments to be separated and was typically between 0.7 and 1.2%.

4.5.3. Isolation of DNA from agarose gels

Following electrophoresis, a DNA fragment was excised from agarose gels under UV transilluminator using a sterile scalpel. The DNA was purified using the NucleoSpin® Extraction Kit according to the manufacturer's instructions.

4.5.4. Extraction with Nucleospin kit

The NucleoSpin® Extraction Kit was used according to the manufacturer’s instruction for the isolation of DNA from PCR reactions and other aqueous DNA solutions

4.5.5. Precipitation of DNA

Ice cold sodium acetate (pH 4.5) was added to a DNA sample to a final concentration of 0.3 M. To this sample 2.5 volumes of ice cold absolute ethanol was added. The sample was incubated at -70°C for 15 min and centrifuged at 12,000 rpm for 15 min at 4°C. The pellet was washed in 70% ethanol, air-dried and re-suspended in an appropriate buffer.

4.5.6. Isolation of plasmid DNA

A single colony of bacteria was used to inoculate 3 ml of Luria Bertani (LB) medium with an appropriate antibiotic and was incubated at 37°C overnight. The overnight culture was centrifuged and plasmid DNA was isolated using the Nucleobond Plasmid isolation Mini kit according to the manufacturer’s instructions. Large scale isolation of plasmid DNA was done from 250 ml of overnight bacterial culture using Nucleobond Plasmid Maxi kit according to the manufacturer’s instructions.

4.5.7. Isolation of total RNA

Before isolation of RNA, all pipettes and working bench were made RNase free using 0.1M NaOH, 1mM ethylenediamine tetracetate (EDTA) and diethyl pyrocarbonate (DEPC) treated H_20. The electrophoresis tanks were washed with 0.5% SDS, DEPC treated H_20 and then with 70% ethanol. Snap frozen material was used for the isolation of RNA. Mammalian cells were directly homogenised through QIAshredder. RNA was isolated from the QIAshredder lysates using RNeasy Mini Kit according to manufacturer's instructions. The integrity of the RNA was proofed by gel electrophoresis of the total RNA and the concentration was measured using the spectrophotometer.

4.5.8. Electrophoresis of total RNA

A 1% agarose gel was prepared in 1X formaldehyde agarose (FA) buffer and allowed to equilibrate in the running buffer for 30 min. Total RNA (4 μl) was mixed with 1 μl of 5X RNA loading buffer, heated at 65°C for 3 min, cooled on ice and loaded on to the gel. The gel was run at 80V to resolve the RNA. Two bands corresponding to the 28 S and 18 S ribosomal RNA, with no smear, was observed confirming the integrity of the RNA.

4.5.9. Determination of the concentration of nucleic acids

The concentrations of DNA and RNA were measured using the Nanodrop Spectrophotometer according to the manufacturer's instructions. The calculations were done by the instrument based of the following formula:

1 unit of absorbance of dsDNA at OD_{260} = 50 µg/ml dsDNA

1 unit of absorbance of RNA at OD_{260} = 40 µg/ml RNA

The OD_{280} was also measured and the ratio between the two ODs indicated the purity of the DNA solution. For pure DNA, 1.8 ≤ absorbance at OD_{260} / absorbance at OD_{280} ≤ 2.0

A value lesser than 1.8 indicated contamination with proteins or with aromatic substances like phenol, while a value greater than 2.0 indicated a possible contamination with RNA.

4.5.10. Restriction analysis of isolated plasmids

In order to verify constructs of plasmids/inserts resulting from cloning, plasmid DNA was isolated and analysed. Independently, transformed bacterial colonies were picked and grown in 5 ml LB/antibiotic overnight. Plasmid DNA was isolated and digested at restriction sites used for cloning of the insert DNA. The digested DNA was then analysed by agarose gel electrophoresis.

4.5.11. Restriction digestion of DNA

DNA was digested at the optimal temperature of restriction enzymes according to the pipetting scheme as described below.

Component	**Amount**
DNA	(0.1 to 5 μg)
10X restriction enzyme buffer	2 μl
10X Bovine Serum Albumin	2 μl
Restriction enzymes	5 to 20 units
HPLC water	up to 20μl

Following digestion, the mixture was heated at 70°C for 15 min to stop enzymatic reaction and purified using the Nucleobond PCR purification kit

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4.5.12. Polymerase Chain Reaction (PCR)

Thermo stable DNA polymerases were used for the amplification of DNA (Saiki et al., 1988; Bej et al., 1991). Two types of PCR amplifications were used in this study: amplification of *A. viteae* tropomyosin cDNA fragments using the standard Taq polymerase and amplification of *O. volvulus* tropomyosin gene from cDNA library using Phusion high fidelity polymerase. The PCRs were set up and performed according to the following schemes.

Reagent	Taq polymerase	Phusion polymerase
Forward primer (10 pmol/ μl)	1 μl	5 μl
Reverse primer (10 pmol/ μl)	1 μl	5 μl
dNTP mix (2mM)	2 μl	2 μl
10X Reaction buffer with $MgCl_2$	2 μl	2 μl
DNA template	10-100 ng	10-100 ng
HPLC water	Up to 50 μl	Up to 50 μl
DNA polymerase	2.5 units	2.5 units

The PCRs were performed according to the following thermal profiles.

Phase		Taq polymerase	Phusion polymerase
Denaturation		94°C, 1 min	98°C, 1 min
Denaturation		94°C, 1 min	98°C, 30 s
Annealing	30 cycles	53 – 60°C, 1min	53 – 60°C, 30 s
Elongation		72°C, 1-3 min	72°C, 1-3 min
Extension		72°C, 10 min	72°C, 10 min

4.5.13. Reverse Transcription PCR (RT-PCR)

Messenger RNA (mRNA) was reverse transcribed to cDNA using SuperScript III Reverse Transcriptase. About 0.5 to 5 μg of total RNA was incubated with 0.5 μg of Oligo dT primer and 1 μl of 10 mM dNTP to a final volume of 10 μl at 65°C for 5 min. The reaction was then placed on ice for 1 min. To this, 2 μl of 10X reaction buffer, 2 μl of 0.1M DTT, 1 μl of RNase Out, 1 μl of Superscript III RT and water to a final volume of 20 μl were added. The reaction was incubated at 50°C for 50 min followed by incubation at 85°C for 10 min to inactivate the enzyme. The synthesized cDNA was used as template for PCR reactions as mentioned above (4.23).

4.5.14. Ligation

DNA fragments were ligated into vectors using the T4 DNA ligase. DNA fragments, PCR products and vectors were restriction digested as mentioned in section 4.22. The purified products, insert and vector were used for ligation. The ligation reaction was set up as follows.

Component	**Amount**
Vector	1/3 molar ratio of insert
10X ligation buffer	2 μl
Insert DNA	3 molar excess of vector
T4 DNA ligase	400 Weiss units
HPLC water	Up to 20 μl

The ligation reaction was incubated at 16°C overnight and 1 to 3 μl were used for transformation of competent *E. coli* cells. For ligation of PCR products into the pGEM-T Easy vector, the same protocol as mentioned above was used. Insert DNA was used in 3 to 5 molar excess of vector and after incubation at 16°C overnight it was used for transformation.

4.5.15. Cloning of A. viteae tropomyosin into pCDNA 3.1+ vector

The *A. viteae* tropomyosin gene sequence was cut out of pBluescript/AvTropo plasmid with restriction enzyme Apa I. Resulting DNA fragment was ligated into pCDNA 3.1+ mammalian expression plasmid. To check if this procedure was successful new recombinant plasmid, amplified and purified from bacteria, was a subject of both PCR and restriction digestion.

The cDNA sequence was subcloned into the Bam HI and Hind III sites of the expression vector pQE30 yielding polypeptides with a six-histidine tag (Qiagen, Hilden, Germany). The recombinant plasmid was transformed into DH5α competent *E. coli* and screened for expression by SDS-PAGE after induction with 1mM IPTG.

4.5.16. Cloning of O. volvulus tropomyosin into pET28b vector

O. volvulus tropomyosin cDNA was isolated from a cDNA library created from female *O. volvulus* RNA by PCR with specific primers and a high fidelity Pfusion polymerase. This resulted in a clone of 855 bp in length and with a deduced amino acid sequence of 284 residues. The clone was named *O. volvulus* tropomyosin and inserted into the pET28b+ expression vector. The resulting construct was used to transform competent bacteria and to express and purify the recombinant protein.

4.6. Microbiological methods

4.6.1. Preparation of competent E. coli

E. coli were streaked on LB agar plates without antibiotics and cultured overnight at 37°C. A single large colony was picked with a sterile toothpick and used to inoculate 125 ml of SOB in a 1-liter Erlenmeyer flask. Flasks were incubated at 18°C with vigorous shaking (220 rpm) and the bacteria grown to an OD of 0.6 (mid-log phase). Bacteria cultures were poured into 50 ml tubes and incubated on ice for 10 min and then centrifuged at 2500x g (3000 rpm). The pellet was resuspended in 40 ml ice-cold transformation buffer (TB), incubated on ice for 10 min and centrifuged as above. The pellet was then carefully resuspended in 10 ml of TB, and DMSO added drop-wise to a final concentration of 7%. The bacterial suspension was incubated for ten minutes on ice, after which 1 ml aliquots were snap-frozen in liquid nitrogen and stored at – 80°C.

4.6.2. Transformation of competent E. coli

Either 10 ng of purified plasmid DNA or 1-3 μl of ligation mixture in a total volume of 20 μl were pipetted into tubes pre-chilled on ice. Competent cells were thawed and 100 μl were dispensed into the tubes on ice. The tubes were flicked gently to mix and incubated on ice for 30 min. The cells were then heat-shocked by heating for exactly 45 s in a 42°C water bath, followed by incubation on ice for 2 min. Room temperature SOC medium (900 μl) was added to each tube on ice. The tubes were then incubated at 37°C for one hour with constant shaking. Antibiotic selective agar plates were plated with 50 to 150 μl of the transformation mixture and incubated overnight at 37°C. Two methods were

routinely employed to identify bacteria colonies that contain the recombinant plasmids or plasmids of interest.

4.6.3. Colony PCR

Bacterial colonies were proofed for successful transformation by colony PCR. The colonies were picked using toothpick, lysed by boiling and introduced into PCR tubes witch contained PCR master mix with the specific primers for the gene. The PCR was carried out as mentioned above with an additional incubation at 95°C for 5 min before the PCR.

4.6.4. Bacteria cultures and long term storage of stocks

Bacteria host strains used in this study included XL 1 Blue, BL 21 (DE3), DH5α and JM109. Bacteria were streaked on LB agar plates and grown overnight at 37°C. For long-term storage of bacteria stock, a single colony was grown in LB overnight and sterile glycerol was added to a final concentration of 10%. Aliquots of 1ml were snap frozen in liquid nitrogen and stored at -80°C.

4.6.5. Expression and purification of protein expressed in E. coli

The pQE and pET expression systems were used for prokaryotic expression of recombinant *A. viteae* and *O. volvulus* tropomyosins, respectively. In these systems, target genes are cloned under the control of the T7 promoter which is not recognised by *E. coli* RNA polymerase. Some *E. coli* host strains (like DH5α and BL21 (DE3)) have a chromosomal copy of the T7 RNA polymerase gene under control of *lacUV5*. Transfer of recombinant plasmids into such hosts results in an IPTG-inducible gene expression system. Both cDNA sequences of *A. viteae* and *O. volvulus* tropomyosin were cloned in such way that a 6 His tag present in plasmids was placed at the C terminal end of genes. pQE30/AvTropo was transformed into DH5α and pET28b/OvTropo was transformed into BL21(DE3) cells.

A single bacterial colony was picked and used to inoculate LB (20 µg/ml Ampicilin or 15 µg/ml Kanamycin) and the culture was grown overnight at 37°C, 200 rpm. The overnight

culture was diluted 1 : 50 in fresh 500 ml of LB medium and further grown at 37°C till an OD_{600} of 0.5-0.6 Protein expression was induced with 1mM IPTG for 4 h. Bacteria were pelleted by centrifugation at 6000 rpm for 15 min at 4°C. Bacterial pellet was suspended in 20 ml of A1 lyse buffer pH 7.9, with 0.05% Triton and lysozyme and kept on ice for 1 h. It was sonicated for 3 min at 30 W and centrifuged at 12,000 rpm for 20 min at 4°C. To purify recombinant protein supernatant was passed through an equilibrated Ni-NTA column (1 ml) on ÄKTA system. The charged column was washed with A2 buffer pH 7.9. The protein bound to the Ni-NTA was eluted in fractions of 500 µl with buffer B, pH 7.9. The eluted protein was dialysed overnight against PBS pH 7.4. The various fractions of washing and elution were analysed by SDS-PAGE and Western blot. The pure protein was purified of LPS contaminant by EndoTrap system sterilized and stored at -20°C for further use.

4.7. Microscopy

Light and fluorescence microscopy were done using a Zeiss Axioplan fluorescence microscope with a blue excitation filter set. For detection of EGFP (enhanced green fluorescent protein) fluorescence in mammalian cells, the transfected COS7 and HeLa cells were suspended in PBS and mounted on slides. For the detection of FITC labelled antibody bound to tropomyosin on *A. viteae* cuticle, the samples were mounted on slides and observed using a confocal laser-scanning microscope (Lieca TCS SP) with an argon/krypton gas laser. The gas laser was set to 50 – 60% and the detection of GFP was done at 488 nm.

4.8. Statistical analyses

The numbers of worms recovered from vaccinated and non-vaccinated *Meriones* in immunisation experiments were compared using the Mann-Whitney U-test at the 95% significance level ($p<0.05$).

5. Materials

5.1. Laboratory equipment

Nanodrop	PeqLab, Germany
Electrophoresis apparatus	Pharmacia Biotech, Freiburg AGS, Heidelberg
Electronic precision weighing instrument BP 3100 S	Sartorius, Göttingen
ELISA-Reader MRX and washing instrument	Dynatech MRW Laboratories, Denkendorf
Incubators	
Warm incubator	Memmert, Schwabach
Shaking incubator	New Brunswick Scientific, Netherland
Glass homogenizer	Braun, Melsüugen
Magnetic stirrer	IKA Labortechnik, Staufen
Microscopes	
Leica DM/R	Leica, Solms
Zeiss Axioplan	Zeiss, Oberkochen
Leica TCS NT	Leica, Solms
Confocal laser-scanning microscope (TCS SP)	Leica, Solma
pH-Meter	WTW, Weilheim
Precision pipettes	Eppendorf, Hamburg
Sterile work bench	Heraeus Christ, Zürich
Thermoblock	Eppendorf, Hamburg
PCR cycler	
Waterbath DC3	Haake, Karlsruhe
Video documentation apparatus E.A.S.Y.RH	Herolab, Wiesloch
Bandelin Sonoplus HD 200 sonicator	Berlin
LSRII	BD Biosciences, Heidelberg
FACS Calibur	BD Biosciences, Heidelberg
Lumi Imager™	Roche Diagnostics, Mannheim
SPOT Synthesizer	Abimed, Langenfeld
Centrifuges	
Cooling centrifuge 4804 R	Eppendorf, Hamburg
Table centrifuge 5415 C	Eppendorf, Hamburg
Large volume cooling centrifuge J2-HS	Beckmann, München

5.2. Primers

The primers were synthesised by TIB-MOLBIOL, Berlin. The restriction enzyme sequences included in the primers are within brackets.

A. viteae tropomyosin

Tropo fw	5′ ATG GAT GCG ATC AAG AAA AAG 3′
Tropo rev	5′ ATA TCC AAA AAG TTC TTG GAA GG 3′

O. volvulus tropomyosin

OvTrBamHI+	5′ CGC GGA TCC G ATG GAT GCG ATC AAG AAA 3′
OvP1	5′ GAT AAG CTT ATG GAT GCG ATC AAG AAA AAG ATG 3′
OvP2	5′ GTA CTC GAG TTA TTT ATC CGT TGC AAT CTT CAA ACG 3′

5.3. Vectors

pEGFP-N1	Clontech, Heidelberg
pcDNA 3.1+	Invitrogen, Karlsruhe
pQE30b	QIAGEN, Hilden
pET28b	Novagen, Darmstadt
pGEMT Easy	Promega, Mannhiem

5.4. E. coli strains

E. coli BL21 (DE3)	Stratagene, Heidelberg
E. coli DH5 α	Promega, Heidelberg
E. coli JM109	Stratagene, Heidelberg
E. coli XL1 Blue	BD Biosciences, Heidelberg
X63-Ag8.653	German Centre for Arthritis Research, Berlin
RBL-2H3	Weizmann Institue, Rehovot, Israel
COS7	Dep.of Virology, Charite, Berlin
HeLa	Dep. of Molecular Parasitology

5.5. Consumables

Dialysis tubing	Serva, Heidelberg
Disposable pipettes (10 and 25 ml)	Costar, Bodenheim
Cryotubes	Greiner, Nürtingen
Nunc Immuno™-Plates, 96 wells	Nunc, Roskilde, Denmark
Microtiterplates (Flat bottom and U-bottom)	Corning Life Science, Schiphol, Netherlands, Greiner, Nürtingen
Pipette tips	Sarstedt, Nürnbrecht Greiner Nürtingen
Pipette tips, RNAse frei	Roth, Karlsruhe
Nitrocellulose Filter Protran BA 83	Schleicher & Schuell, Dassel
Microscopic slides, SuperFrost®*/Plus	Menzel-Gläser, Braunschweig
Petriplates, ø 14 cm und 145 cm	Greiner, Nürtingen
Polypropylene centrifuge tubes (15 und 50 ml)	Greiner Nürtingen
Sterile filters	Schleicher & Schuell, Dassel
Whatman-Paper	Schleicher & Schuell, Dassel
Cell culture flasks	Biochrom, Berlin
Cell scrapers	Biochrom, Berlin
Cell culture plates	Costar, Bodenheim
Fuchs-Rosenthal chamber	Roth, Karlsruhe
NeuBauer chamber	Roth, Karlsruhe
Microhaematocrit capillaries	Roth, Karlsruhe
Microcapillaries	Roth, Karlsruhe
Cryovials	Cellstar, Frickenhausen
Microscopic slides	Roth, Karlsruhe

5.6. Reagents

Acrylamide-Bisacrylamide-Solution, Rotiphorese Gel 30	Roth, Karlsruhe
Acetic acid	AppliChem, Darmstadt
Agar	Roth, Karlsruhe
Agarose	Roth, Karlsruhe
Ammonium persulfate (APS)	Serva, Heidelberg
Ammonium chloride	Roth, Karlsruhe
Antibiotics	AppliChem, Darmstadt
Bromophenol blue	Merck, Darmstadt
5-Bromo-4-chloro-3-indolylphosphate disodium salt (BCIP)	Roth, Karlsruhe
5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal)	AppliChem, Darmstadt
Bovine serum albumin (BSA)	Sigma, Deisenhofen
Chloroform	Merck, Darmstadt
chemiluminescence substrate	Pierce, Bonn
Citric acid	Roth, Karlsruhe
Diethylpyrocarbonate (DEPC)	Roth, Karlsruhe
N,N-Dimethylformamide (DMF)	AppliChem, Darmstadt
Dimethylsulfoxide (DMSO)	Roth, Karlsruhe
DNA Ladder 1 kb	Rapidozym, Berlin
dNTPs	Rapidozym, Berlin
epibromohydrin	Sigma, Munich
Ethylenedinitrotetraacetic acid (EDTA)	AppliChem, Darmstadt
Ethanol pure	AppliChem, Darmstadt
Ethidiumbromide	AppliChem, Darmstadt
Fetal calf serum	Biochrom, Berlin
Fugene 6	Roche, Mannhiem
Glucose	AppliChem, Darmstadt
L-Glutamine	Biochrom, Berlin
Glutaraldehyde	Sigma, Steinheim
Glycine	AppliChem, Darmstadt
Hydrogen peroxide 30%	Roth, Karlsruhe
Mouse IL-6	Roche, Munich
Isopropyl-β-D-thiogalactopyranoside (IPTG)	AppliChem, Darmstadt
Lysozyme	AppliChem, Darmstadt
Magnesium chloride	AppliChem, Darmstadt
Maleic anhydride	Sigma, Steinheim
Maltose	AppliChem, Darmstadt
Methanol, analytical	AppliChem, Darmstadt
2 C 180 Methylene blue Unna	ChromaGesellschaft, Münster
Monohydrate-Citric acid	Roth, Karlsruhe
3-Morpholinopropanesulfonic acids (MOPS)	AppliChem, Darmstadt
p-Nitrobluetetrazoliumchloride (NBT)	Roth, Karlsruhe
p-Nitrophenyl-N-Acetyl-ß-D-Glucosamine	Sigma, Deisenhofen
p-Nitrophenyl Phosphate Disodium	Sigma, Munich
Ni-NTA-Matrix	QIAGEN, Hilden
Papain	Sigma, Steinheim
Paraformaldehyde (PFA)	Merck, Darmstadt
Penicillin/Streptomycin (10000 U/10000 μg/ml)	Biochrom, Berlin
Protein Marker, Broad Range prestained	New England Biolabs, Schwalbach
Phenylmethylsulfonylfluoride (PMSF)	Fluka, Steinheim
Phosphatase substrate tablets	Sigma, Steinheim
D(+)-Saccharose	AppliChem, Darmstadt
Sodium acetate	AppliChem, Darmstadt
Sodium borohydrate	Roth, Karlsruhe

Sodium chloride	AppliChem, Darmstadt
Sodium citrate	Roth, Karlsruhe
di-Sodium hydrogen phosphate	AppliChem, Darmstadt
Sodium hydroxide	AppliChem, Darmstadt
Sodium meta peroxide	AppliChem, Darmstadt
Sodium hypochloride	Roth, Karlsruhe
Sodiumdodecyl sulfate(SDS)	AppliChem, Darmstadt
Squalane	Roth, Karlsruhe
Sulfuric acid	AppliChem, Darmstadt
TEMED	Roth, Karlsruhe
Teepol	Serva, Heilderberg
3,3´,5,5´-Tetramethylbenzidine (TMB)	Sigma, Steinheim
4,7,10-trioxa-1,13-tridecanediamin	Sigma, Steinheim
Tris Base	AppliChem, Darmstadt
Triton X-100	Serva, Heidelberg
Trypan bleue	AppliChem, Darmstadt
Trypton	AppliChem, Darmstadt
Trypsin-EDTA	Biochrom, Berlin
Tween 20	AppliChem, Darmstadt
Tyrode salts	Sigma, Steinheim
Uranyl Acetate	Merck, Darmstadt
Urea	AppliChem, Darmstadt
Yeast extract	AppliChem, Darmstadt

5.7. Commercial Kits

BCA Protein Assay Kit	Pierce, Bonn
Gel drying-Kit	Promega, Heidelberg
NucleoSpin® Mini Plasmid Kit	Macherey & Nagel, Düren
NucleoBond® Midi Plasmid Kit	Macherey & Nagel, Düren
NucleoSpin® Extraction Kit	Macherey & Nagel, Düren
NucleoTrap® Gel Extraction Kit	Macherey & Nagel, Düren
pGEM®-T Easy Vector System I	Promega, Mannheim
RNeasy® Mini Kit	QIAGEN, Hilden
QIAshredder spin columns	QIAGEN, Hilden
EndoTrap	Profos, Regensburg
OptEIA mouse IL-4 kit	BD Pharmingen,
OptEIA mouse IL-10 kit	BD Pharmingen,
OptEIA mouse INFχ kit	BD Pharmingen,
BioTrap BT 1000 device	Schleicher & Schuell, Dassel

5.8. Enzymes

DNA-Restriction enzymes	New England Biolabs, Schwalbach
Lysozyme	AppliChem, Darmstadt
Phusion polymerase	Finnzyme, Espoo, Finland
Taq-Polymerase	Rapidozym, Berlin
SuperScript III Reverse Transcriptase	Invitrogen
T4 DNA-Ligase	New England Biolabs, Schwalbach

5.9. Solutions, mediums and buffers

Dulbeccos MEM medium	Biochrom, Berlin
MEM-EARLE medium	Biochrom, Berlin
RPMI 1640 medium	Biochrom, Berlin
Eagle's minimum essential medium	Biochrom, Berlin
Tyrode's buffer	Sigma, Munich
TE buffer	10mM Tris pH 7.4 1mM EDTA pH 8

5.9.1. Agarose gel electrophoresis buffers

6 x Agarose-Loading buffer	40% D(+)-Saccharose 0.005% Bromphenolblue
Ethidiumbromide-solution	1% in H_2O
10 x FA-Gel-buffer	200 mM MOPS 50 mM Sodium acetate 10 mM EDTA in RNase-free H_2O pH 7
1 x FA-Gel running buffer	1 x FA Gel-buffer 0.74% Formaldehyde in RNase-free. H_2O
5 x RNA Loading buffer	0.16% ges. Bromphenol blue solution 4 mM EDTA, pH 8 2.7% Formaldehyde 20% Glycerol 30.1% Formamide 4 x FA Gel buffer in RNase-free H_2O
TAE-buffer	40 mM Tris-HCl 1 mM EDTA 0.11% Acetic acid pH 8

5.9.2. Bacterial culture medium

IPTG stock solution	1 M IPTG in dest. H_2O
LB-Medium	0.5% NaCl 1% Trypton 0.5% Yeast extract in autoclaved H_2O, pH 7.5
LB-Agar	LB-Medium 1.5% Agar autoclaved
SOB-Medium	0.05% NaCl 0.5% Yeast extract 2% Tryptone 2,5 mM KCl 10 mM $MgCl_2$

	Autoclaved
SOC-Medium	SOB-Medium 20 mM Glucose, sterile filtered
TB-Buffer	10 mM PIPES 15 mM $CaCl_2$ 250 mM KCl pH 6.7 55 mM $MgCl_2$ Sterile filtered
X-Gal	2% in DMF

5.9.3. Antibiotics

Ampicillin	100 mg/ml in. H_2O in cultures: 1/1000
Chloramphenicol	34 mg/ml in. H_2O in culture: 1/1000
Kanamycin	25 mg/ml in H_2O in culture 1.5/1000
Tetracyclin	20 mg/ml in Ethanol in culture: 1.5/1000

5.10. Protein and Immunochemistry
5.10.1. SDS-PAGE

APS-Stock solution	10% APS in H_2O
Coomassie-Staining solution	10% Acetic acid 20% Ethanol 0.2% PhastGel®BlueR
Coomassie-Destaining solution	10% Acetic acid 20% Ethanol
Running buffer	190 mM Glycine 25 mM Tris-HCl 0.1% SDS
Solution L	1.5 M Tris-HCl 0.4% SDS pH 8.4
Solution M	0.5 M Tris-HCl 0.4% SDS pH 6.8
2 x Loading buffer	10% Glycerin 5% β-Mercaptoethanol 3% SDS 100 mM Tris-HCl 0.02% Bromphenolblau

5.10.2. Solutions for SDS-Polyacrylamide gel

	6% (Stacking gel)	**10% (Resolving gel)**	**12% (Resolving gel)**
Acrylamide (30%)	0.375 ml	1.65 ml	2 ml
H_2O	1.5 ml	2.05 ml	1.75 ml
Solution L	-	1.25 ml	1.25 ml
Soluition M	0.625 ml	-	-
TEMED	2.5 µl	5 µl	5 µl
APS (10%)	25 µl	50 µl	50 µl

5.10.3. Western Blot

AP-Detection buffer	100 mM Tris-HCl 100 mM NaCl 5 mM $MgCl_2$ pH 9.5
BCIP-Stock solution	5% in DMF (100%)
NBT-Stock solution	5% in DMF (70%)
PBS	171 mM NaCl 3.4 mM KCl 10 mM Na_2HPO_4 1.8 mM KH_2PO_4 pH 7.4
Transfer buffer	48 mM Tris-HCl 39 mM Glycine 0.037% SDS 20% Methanol
TBS-T	TBS 0.05% Tween 20 pH 7.4

5.10.4. Immunostaining

Wash solution	0.1 M Tris-HCl, pH 7.5 and PBS / 0.05% Tween, pH 7.4
Blocking solution	10% FCS in PBS pH 7.4
Fixing solution	2.5% Paraformaldehyde in PBS, pH 7.3 and 4% formaldeyde in PBS
Mowiol mounting medium	7 g Glycerol 2.4 g Mowiol 6 ml H_2O Mix at RT for 2 h 12 ml 0.2 M Tris-HCl pH 8.5 1 h at 50 °C

5.10.5. Protease inhibitors

Solution I	100 mM PMSF in Isopropanol
Solution II	100 mM EDTA (Titriplex III) 100 mM ε-Aminocapric acid 100 mM Benzamidine

5.10.6. Ni-NTA-Affinity chromatography

Lysis buffer (A1)	20 mM Tris-HCl 500 mM NaCl 5 mM Imidazol 0.1 % Triton X-100 Lysozyme pH 7.9
Wash buffer (A2)	20 mM Tris-HCl 500 mM NaCl 60 mM Imidazol pH 7.9
Elution buffer (B)	20 mM Tris-HCl 500 mM NaCl 60 mM Imidazol pH 7.9

5.10.7. mAb-Affinity chromatography

Lysis buffer	PBS, pH 8
Wash buffers	PBS pH 6.3 PBS pH 5.9
Elution buffer	PBS pH 4.1

5.10.8. ELISA

Coating buffer	13 mM Na_2CO_3 35 mM $NaHCO_3$ pH 9.6
Blocking solution	3% BSA in PBS
Phosphate Citrate buffer (0.1M)	25.7 ml 0.2 M Na_2HPO_4 24.3 ml 0.1 M Citric acid pH 5
Phosphatase substrate solution	1 Tablet. Phosphatase substrate 20 ml coating buffer 0.1 mM $MgCl_2$
Stopping solution	for AP-conjugate: 0.1 M EDTA for POX conjugate: 1M H_2SO_4
TMB-Substrate solution	1 Tablet TMB 5 ml H_2O 0.05 M Phosphate-Citrate-buffer, pH 5 0.006% H_2O_2
Wash buffer	PBS/0.025% Tween, pH 7.4

5.10.9. Antibodies

24-4 mAb anti-chitinase	Dept. of Molecular Parasitology
1E5 mAb	Dept. of Molecular Parasitology
TM311 mAb anti-chicken gizzard tropomyosin	Sigma, Munich
Rat anti-mouse IgE - Pox	Serotec, Dusseldorff,
Goat anti-mouse IgG - Pox	Jackson ImmunoResearch, West Grove, USA
Goat anti-mouse IgM	Jackson ImmunoResearch, West Grove, USA
Goat anti-mouse IgG	Jackson ImmunoResearch, West Grove, USA
Rabbit anti-mouse IgG1	Rockland, Gilbertsville, USA
Rabbit anti-mouse IgG2a	Rockland, Gilbertsville, USA
Rabbit anti-mouse IgG2a	Rockland, Gilbertsville, USA
Rabitt anti-mouse IgG3	Rockland, Gilbertsville, USA
Goat anti-mouse IgG – AP	Dianova, Hamburg
Goat anti-mouse IgG –FITC	Dianova, Hamburg
Goat anti-mouse FcγR	German Centre for Arthritis Research, Berlin
Rat anti-mouse CD11b (FITC)	BD Pharmingen,
Rat anti-mouse F4/80 (Cy5)	German Centre for Arthritis Research, Berlin
Rat anti-mouse Gr1 (Biotin)	German Centre for Arthritis Research, Berlin
Goat anti-mouse IgG (SA-PeCy7)	German Centre for Arthritis Research, Berlin

5.11. Immunization of M. unguiculatus

STP adjuvant	0.4% Tween 20 1% Synperonic 10% Squalane in PBS
Alum adjuvant	Reheis, Dublin, Ireland
Aluminium phosphate (AdjuPhos)	BrentagBiosector, , Denmark
Xylazin	Bayer Vital GmbH, Leverkusen
Ketamin	Pharmacia GmbH, Karlsruhe

5.12. Software

FlowJo (FACS)	Tree Star, Inc. Ashland, USA
LISA (SPOT synthesizer)	Jerini AG, Berlin
MacVector 7.2 (sequence alignment)	Accelrys, USA

6. Abbreviations

APS	Ammonium peroxidisulfate
Amp	Ampicillin
AP	Alkaline phosphatase
bp	Base pairs
BCA	2,2´Bicinchoninic acid
BCIP	5-Bromo-4-chloro-3-indolyphosphate disodium salt
BSA	Bovine serum albumin (Fraction V)
cDNA	Complementary DNA
CTAB	Cetyltrimethylamoniumbromide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediamino tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EtBr	Ethidium bromide
EtOH	Ethanol
HPLC	High performance liquid chromatography
HAT	Hypoxanthine, Aminopterin, Thymidine
HT	Hypoxanthine, Thymidine
IPTG	Isopropyl-thio-ß-D-galactopyranoside
IU	International unit
kb	Kilo base
kDa	Kilo Dalton
L_3	Third stage larvae
LB	Lauria Bertani
2-ME	ß-Mercaptoethanol
mAb	Monoclonal antibody
mf	Microfilariae
mRNA	Messenger RNA
NBT	Nitroblue tetrazoliumchloride
NC	Nitrocellulose
OCP	Onchocerciasis Control Program
OD	Optical density

ON	Overnight
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Peritoneal exudate
PEG	Polyethyleneglycol
PIPES	1,4-Piperazinediethane sulfonic acid
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RT	Room temperature
SDS	Sodium dodecylsulfate
TAE	Tris-acetate-EDTA
TB	Transformation buffer
TBS	Tris-buffered saline
TE	Tris-EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
TRIS	2-Amino-2-hydroxymethyl-1,3-propandiol
U	Units
UV	Ultraviolet
X-gal	5-Bromo-4-chloro-3-indoxyl-ß-D-galactoside

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